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Investigations on the significance of the gastrointestinal flora for the immune system of chickens

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To my Parents and to my family
(Hanan, Noorhaan, Nirvan and Nada)
with all love and gratitude

Abbreviations

AA, C20:4n-6	Arachidonic acid
Ab	Antibody
AC	Anaerobic cultures
ADCC	Antibody-dependent T cell-mediated cytotoxicity
APP	Acute phase proteins
AV	Average value
BC	Bacitracin
Bdello	*Bdellovibrio*
BPI	Bactericidal permeability increasing factor
BF	Bursa of Fabricius
BSA	Bovine serum albumin
BW	Body weight
C. albicans	*Candida albicans*
CE	Competitive exclusion
Cfu	Colony forming unit
C-group	Control group
CL	Colistin
C. perf.	*Clostridium perfringens*
CRP	C-reactive protein
DF	Dietary fiber
DNA	Deoxynucleic acid
E. coli	*Escherichia coli*
EFA	Essential fatty acid
Eh	Oxidation-reduction potential
ELISA	Enzyme linked immunosorbent assay
E. maxima	*Eimeria maxima*
Endo.	Endotoxin
ER	Enramycin
EU	Endotoxin Unit
FOS	Fructooligosaccharide
g	Gram
gram+	Gram-positive bacteria
GALT	Gut-associated lymphoid tissue
GIT	Gastrointestinal tract

G negative	Gram-negative
H	Herd
h	Hour
HF	High fat
HM	Hepesbuffer with Mg^{2+}
Ig	Immunoglobulin
kg	Kilogram
J. A.	Jerusalem artichoke
LAL	Limulus-Amoebocyte-Lysate
LB	Lactobacilli
LD	Lactose derivatives
LF	Low fat
LPS	Lipopolysaccharid
L. reuteri	*Lactobacillus* reuteri
LT	Leukotrienes
Min	Minute
MJ	Metabolisale energy
ml	Milliliter
NDO	Nondigestible oligosaccharides
nm	nanometer
P	Pancreas
PC-BP	Phosphoryl choline binding protein
Pfu	Plague forming unit
pH	Logarithm of the hydrogen ion concentration
PG	Prostaglandins
PNA	P-nitroaniline
ppm	Parts per million
PUFA	Polyunsaturated fatty acids
RES	The reticuloendothelial system
RNA	Ribonucleic acid
RSE	Reference standard endotoxin
RU	Relative unit
±s	Standard deviation
SC	Secretory component
S. Enteritidis	*Salmonella* Enteritidis
E. faecalis	*Enterococcus* faecalis
SigA	Secretory IgA
SPF	Specific pathogen free
SRBC	Sheep red blood cells
S. Typhimurium	*Salmonella* Typhimurium

TBC	Total bacterial count
TNF	Tumour necrosis factor
mM	Millimol
U	Unit
VFAs	Volatile fatty acids
W	Week
YP-media	Yeast-peptone media
µg	Microgram
µl	Microliter

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1 Introduction

The intestine of newly hatched chicken is devoid of bacteria. In the first few hours to days of life, the normal microflora that inhabits the intestine become established (OCHI et al. 1964). Intestinal microflora functions to break down ingested food produce some vitamins and most importantly provide a natural barrier to harmful bacteria that enter the host. In the days when chicks were hatched under a hen, the bacteria shed in the faeces of the healthy adult hen provided the inoculums for the establishment of a similar microflora in the chicks. With the advent of modern incubation, the chick or poult is exposed to the first bacteria in the incubator, chick box, and litter of the poultry house.

The normal microflora of the intestinal tract is made up of a diverse population of bacteria. Some of these bacteria are anaerobic (grow without oxygen), some are aerobic (oxygen dependent) and some are inbetween (facultative anaerobes or microaerophilic). All of these bacteria are in competition for survival. They compete for attachment sites and nutrients from the ingesta passing through the intestine. Each species has specific requirements for growth and is affected by relative acidity or alkalinity (pH) of their environment and by products produced by neighboring bacteria. One of the now recognized advantages of this early gut colonization is that, by occupying all the available receptor sites in the gut, undesirable bacteria are excluded because they are unable to compete existing flora. This is what is now known as ‘competitive exclusion’ (CE) (RANTALA and NURMI 1973).

Antibiotics have been used in livestock and poultry production since the 1950's. Drugs sometimes are administered in relatively large (therapeutic) doses to treat sick animals. The use of smaller (subtherapeutic) amounts of antibiotics in animal feeds to prevent or reduce the incidence of infectious diseases and to improve feed efficiency and animal growth is more common. If subtherapeutic uses of all antibiotics were banned, producers would experience slower animal growth rates and more disease caused deaths. Production costs would rise substantially. Higher production costs would mean an increase in the cost of meats, particularly pork and poultry.

The use of antibiotics as growth promoters may lead to an increase in *Salmonella* colonization and increase in antibiotic resistance. It was reported that the use of antibiotics such as oxytetracycline can cause an inhibition of the immune system (FORSGREN and GNARPE 1973). Furthermore, the feeding of chlorotetracycline resulted in increased shedding of salmonellae and enhanced the severity of disease in animals (DEY et al. 1978). In the long-term administration with the antibiotics, the intestinal microflora showed significant changes mainly over a period from 1 to 3 weeks of age. It is well known that a microflora is established 2 or 3 weeks after hatching. Antibiotics contained in feed may affect the establishment of intestinal microflora in broiler chicken.

Table 1. Common used antibiotics and their mode of action (KÖHLER 1988)

Type of antibiotic	Type of effect	Spectrum of effect	Mechanism of action
Chinoxaline (Carbadox / Olaquindox)	Bactericidal	Gram +	Inhibition of DNA-Synthesis
Ionophore (Monensin / Salinomycin)	Bactericidal	Gram +	Affects permeability of cell membrane
Macrolide Tylosin / Spirarnycin)	Bacteriostatic	Gram +	Inhibition of protein synthesis
Phosphoglycolipid (Flavophospholipol)	Bacteriostatic	Gram +	Inhibition of cell wall synthesis
Peptolid (Virginiamycin)	Bacteriostatic	Gram +	Inhibition of protein synthesis
Polypeptide (Zinc-Bacitracin)	Bactericidal	Gram +	Inhibition of cell wall synthesis
Glycopeptide (Avoparcin)	Bactericidal	Gram +	Inhibition of cell wall synthesis

Common sense and knowledge of bacterial genetics tell us that we need to monitor the emergence of resistance and only give antibiotics to animals when it is therapeutically rational and cost-effective to do so. Resistant bacteria can be generated in animals, transferred to humans and amplified to become a major human problem. These bacteria may either cause disease in humans or transfer their resistance genes to normal flora that may later become pathogenic. Indiscriminate or prolonged use of antibiotics can destroy normal body flora, and depress the immune system and create resistant pathogens. The environment of the intestine can be altered by the composition of the diet of the bird or by disease. As an example of the effect of diet on gut microflora, researchers have demonstrated that the colonization of some pathogens can be inhibited by the feeding of complex sugars, like mannose and lactose. Good food hygiene will slow down the transfer rate but will not eliminate the transfer of resistance. Antibiotics should therefore be given to animals only when necessary and for the shortest effective duration.

Finally, antimicrobial agents should not to be used for growth promotion in food-producing animals if they are used in human, these drugs have the downside of potentially causing microbes to become resistant to drugs used to treat human illness, ultimately making some human sicknesses harder to treat. About 70 percent of bacteria that cause infections in hospitals are resistant to at least one of the drugs most commonly used to treat infections.

After recommendations of the World Health Organization, there is a systematic approach toward replacing antimicrobial growth promoters with safer nonantimicrobial drug alternatives. The European Union countries entered this process in December 1998 when four growth promoters

(tylosin, spiramycin, bacitracin and virginiamycin) were banned because of their structural relatedness to therapeutic antimicrobial drugs used for humans.

Fructooligosaccharides reportedly can be substituted for subtherapeutic levels of antibiotics to enhance the growth and production efficiency of broilers (AMMERMAN et al. 1988a, b, 1989). Recent researches have shown an important physiological action for inulin (GIBSON 1999, ROBERFROID 2000). Like some pectins and fructooligosaccharides, inulin is a preferred food for the lactobacilli in the intestine and can improve the balance of friendly bacteria in the bowel. Recent animal research also shows that inulin prevents precancerous changes in the colon (REDDY, 1997). Therefore the aims of this work were to explain

(1) What is the effect of inulin of Jerusalem Artichoke (J. A.) and linseed on the intestinal colonization of naturally infected chicks with *Salmonella* (*S*.) Enteriditis?

(2) What is the effect of inulin (J. A.) and linseed on the intestinal flora, body weight and on the immune status of SPF (specific pathogen free) chicks?

(3) What is the effect of inulin (J. A.) on the intestinal flora, endotoxin and PC-BP blood levels and on the immune status of broiler chicken?

(4) What is the dynamic of the intestinal flora of broilers and breeders' chicken in relation to endotoxin, phosphoryl choline-binding protein (PC-BP) blood levels, bursa of Fabricious (BF)/body weight (BW) and pancreas (P)/BW ratios?

2 Review of Literature

2.1. General anatomy and features of gastrointestinal tract of birds

2.1.1 Morphology of the gastrointestinal tract and how it works

The morphology of the gastrointestinal tract (GIT) (figure 1) varies substantially among birds.

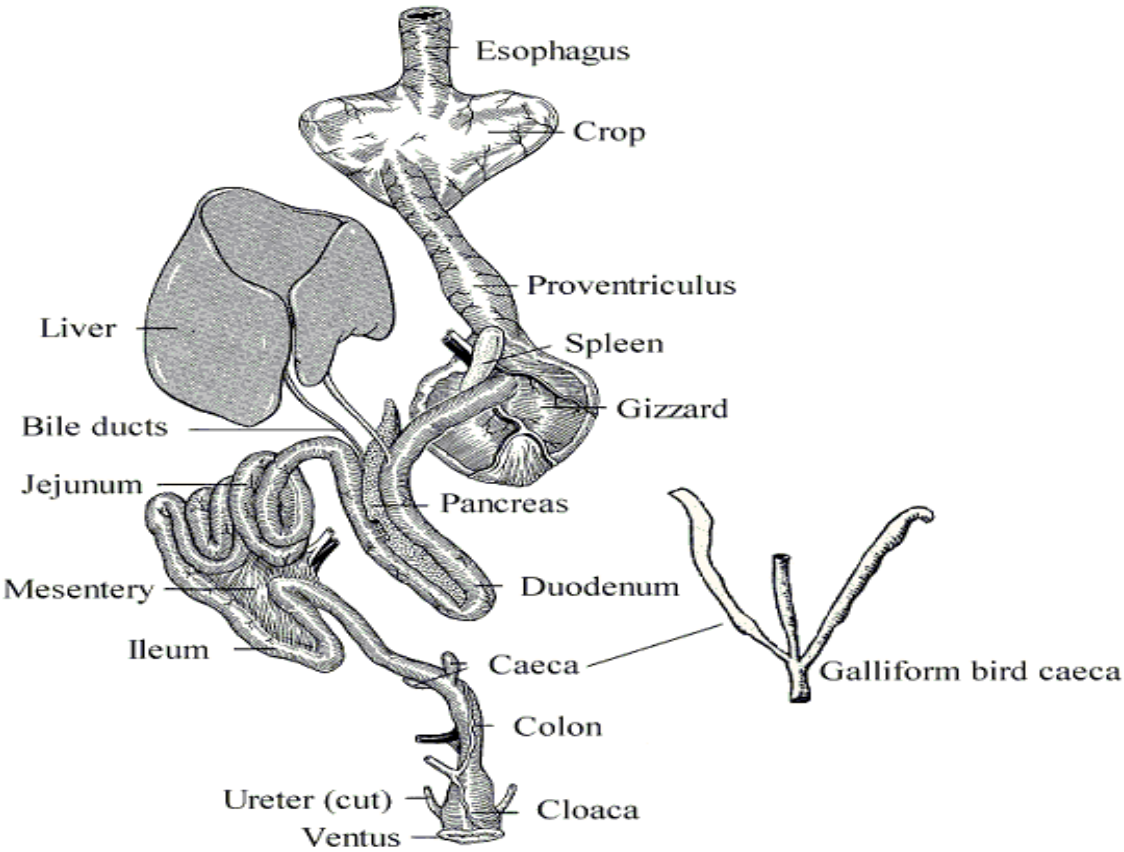


Figure 1. Gastrointestinal tract of the galliform birds (after PROCTOR and LYNCH 1993)

The design and function of the digestive system is based upon the bird's extraordinary need for nutrition and energy. The gastrointestinal tract is faced with the problem of transforming ingested food into an utilizable form of nourishment and energy for billions of cells. To visualize the size of the task, consider this example: a small bird may eat 25% of his weight in food daily. With a 20 gram canary (two-thirds of an ounce), that would be 5 grams of food. Not much volume in the palm of our hand, but compared proportionally to man, a 150-pound person would be eating 37 pounds of food. The question arises immediately of how a bird is capable of handling this volume of food? And further, how could the digestive system begin to convert this bulk to an utilizable form fast enough to provide for the energy demands. Refinement of the digestive system specifically for the purpose of processing proportionally large volumes of food is seen in the production line design of the gastrointestinal tract. The bird has two stomachs. The first one adds digestive juices to the food

as it passes through on its way to the second stomach, which rapidly grinds the food into fine particles and sends it on to the small intestine. By having a crop as the reservoir for food, it can deliver small amounts of ingest continually to the stomach, where it can be rapidly processed. This makes for efficiency, as it enables a relatively constant process of digestion to take place. If the bird had teeth to perform these grinding processes instead of the gizzard, the bird would spend a long time chewing its food, which would expose it to dangers that are avoided by its bolting the food down and flying to safety. Also, it is much more efficient to have the gizzard grind food for hours in a continuous action, as the quantity of food in proportion to the size of the animal is relatively great. As food leaves the gizzard and passes into the intestines, it is mixed with digestive juices from the liver, pancreas, and the wall of the intestine. Bile from the liver and pancreatic juice act to neutralize the acid from the stomach and to emulsify fats in preparation for further digestion. The pancreatic juices digest proteins, fats and carbohydrates, once the food is digested, it is absorbed by the lining of the intestine, passed on into the blood stream, and distributed through the body. The whole process must be synchronized and efficient, as the muscular activity of the intestinal tract propels the food through the intestine rapidly. A starling will have food pass through its digestive tract in twenty minutes, and in a parakeet, it takes less than two hours. The relatively small amount of faeces in the droppings indicates the thoroughness of the whole digestive process. The digestive system is functioning under this intensity because the bird has extraordinary demands for energy. Food, the fuel for the body, is burned at a much higher rate than for any other living thing. Because energy is used rapidly, a bird must constantly depend upon food as a source of fuel. Fat reserves cannot be converted rapidly enough to energy to be of any importance. Any disturbance of the gastrointestinal tract which interferes with food digestion or absorption can cause the bird to starve to death in a few days. The alimentary canal has many adaptations and unusual features compared to mammals. One of these, the cloaca, evolved to eliminate the need for a urinary bladder and colon. An exchange of an organ to replace two is good efficiency. To lighten the load-as needed in flight-the cloaca collects only a small volume and then empties, thus accounting for the many droppings which a bird has. Since the cloaca has an influence on the arrangement and size of the droppings, knowing its structure makes interpretation of droppings easier. The faeces enter from the large intestine on the bottom, the urine enters from the ureters (tubes leading from the kidneys) at the top. When the droppings are passed, the faeces fall first, with the urine (white urates and fluid urine) coming second. In many cases, this allows the urine to cover the faeces. A fold from the top of the cloaca separates the terminal part of the large intestine from the area where the urine is deposited. With this arrangement, at times urine is passed as a dropping without faecal material. The relationship between the structure of the GI-tract and diet is widely studied; the development of the caeca and systematic position usually has no correlation, with one exception. In grouse (Tetraonidae) the long caeca are believed to be related to the high fibre content of their diet (MCLELLAND 1989). The seasonal variation in the length of the small intestine and caeca of the Tetraonid birds is known to reflect changes in their diets (GASAWAY 1976a, PULLIAINEN and TUNKKARI 1983).

Diet composition, at least increased fibre content in food, is assumed to increase the gut and gizzard size in some galliform species (PAGANIN and MENEGUZ 1992, STARCK and KLOSS 1995) and also in some waterfowl (KEHOE and ANKNEY 1985). The most spectacular example of the impact of hand-rearing on the gut is the disparity between wild and hand-reared birds of the same species. Hand-reared birds have lighter gizzards and shorter caeca and small intestine than their wild counterparts at least in the red grouse (MOSS 1972), the willow grouse (HANSSEN 1979a), the pheasant (MAJEWSKA et al. 1979), the mottled duck Anas fulvigula (MOORMAN et al. 1992), and the grey partridge (PUTAALA and HISSA 1995). Feeding of captive willow grouse with commercial chicken food is known to generate a gut microflora similar to the one in domestic fowl Gallus domesticus, and unlike the one in wild willow grouse (HANSSEN 1979b). The adjustment time of the GI to new feeding conditions may range from weeks to months (MOSS 1989 and REDIG 1989).

2.1.2 Components of the normal intestinal flora of chicken

OCHI et al. (1964) studied the development of the normal intestinal flora from chicks till hens. The chicks that had just hatched, were quite germ - free at all levels of intestinal tract. At 6-12 hours after hatching a few bacterial groups (enterococci, *Enterobacteriacae*, micrococci and corynebacteria) were often detected, but they yet remained only in smaller counts. A significant increase in enterococci or enterobacteria was observed in caecum at 12-36 hours after hatching and these organisms reached almost same bacterial count as those after feeding. At this time, however, only a slight increase of them was observed in the other levels of the tract. These findings continued till the first feeding. A rapid increase of the total bacterial counts occurred in the duodenum, middle intestine and rectum after the first feeding and the counts reached the highest value within the first 24 hours after feeding (10^8-10^9 / g faecal content). The constitution of the flora in all sites was similar to those before feeding. Micrococci, clostridia and bacteroides were relatively increased. As the chicks grew older, the flora changed gradually. It was found that the flora of the duodenum, middle intestine and rectum become established in chicks 9-13 days after feeding and that the flora of the caecum, however, was rather later, i.e. in chicks 25-32 days after feeding, the established flora was as follows:

In the duodenum lactobacilli were the most predominant bacteria (10^5-10^7 /g faecal content), making up in most cases 100 % of the total flora. The flora of the middle intestine showed in a slightly greater count and per cent of enterococci and enterobacteria than those of the duodenum. *Bacteroides* and bifidobacteria could not be proved in the duodeum and middle intestine, with a few exceptions. The total bacterial count of the caecal contents ranged from 10^9-10^{10} /g faecal content. The caecal flora was characterized by generally *Bacteroides* and bifidobacteria were predominant, and moreover enterococci (10^6-10^7 /g faecal content) and enterobacteria (10^5-10^7 /g faecal content) were constantly present. SMITH and CRABB (1961) studied the development and composition of the faecal flora during the first few weeks of life in different animal species. They found that, by the end of the first day, large numbers of *E. coli*, clostridia, and enterococci were present, and with another day they were joined by *Bacteroides* and lactobacilli, these two kinds of bacteria soon

constituting the greater part of the faecal flora. As they grew older, considerable quantitative and qualitative differences developed, until after several months the bacterial flora of the faeces of the different species were grossly dissimilar from each other, as well as from those of the very young of all species.

Table 2. The numbers of viable bacteria found in the faeces of ten adult chickens (BARNES et al. 1980)

Logarithm of viable counts per g faeces				
E. coli	***C. perfringens***	***Enterococci***	***Bacteroides***	***Lactobacillus***
6.6	2.4	7.5	0	8.5
(4.0-7.6)	(0-4.4)	(0-8.7)	(0-9.0)	(6.5-9.1)

The caeca of newly hatched chicks obtained either from commercial hatcheries or from a carefully controlled experimental hatchery have been shown to contain high numbers of a variety of microorganisms. Faecal enterococci, clostridia, enterobacteria, pediococci, and occasionally *Pseudomonas aeruginosa* have all been isolated, but never lactobacilli. After the chick has been on feed for 1 day, the numbers of lactobacilli in the crops and caeca are quite variable, by the 3rd day, however, large numbers are present throughout the alimentary tract (BARNES et al. 1980).

Table 3. The types and the numbers of bacteria present at three different sites in the intestine of normal chicken (TIMMS 1968)

Organism	**Log $_{10}$ Viable count (mean and range) of organisms per g contents**		
	18-day-old	**7-week-old**	**5-Month-old**
C. perfringens	2.6	2.5	2.8
	(1.7-4.0)	(1.7-4.0)	(N-3.7)
E. coli	8.5	6.7	6.9
	(7.7-9.0)	(6.0-7.2)	(6.0-7.7)
Enterococci	7.9	6.8	6.7
	(6.5-8.5)	(6.0-7.4)	(5.3-7.5)
Lactobacillus	9.7	8.5	9.2
	(8.7-10.8)	(7.3-9.0)	(8.0-9.3)
Bacteroides	8.8	8.8	8.2
	(7.7-9.2)	(7.0-9.5)	(7.0-9.3)

N= no viable organisms found in 0.02 g of caecal contents (i.e. Log $_{10}$ viable count per g < 1.7)

The clostridia were never present as a major part of the anaerobic flora. Clostridia are regularly found at 10^2 to10^4 /g, but were isolated from the 2-week birds at about 10^9 /g. SMITH (1961) previously showed that feeding a high protein (meat and bone) to young chicken encouraged the growth of *C. perfringens*. In the small intestine the typical adult flora becomes established within

the first 2 weeks after hatching but the caecal flora takes much longer to develop. Although 10^{10} to 10^{11} bacteria/gram are present in the caeca from the first day onward, they initially consist almost entirely of faecal enterococci and coliform bacteria, although clostridia and bacilli are also sometimes found in considerable numbers. The numbers of lactobacilli increase until after about 4th day, when they remain at about 10^8 to 10^9 / g and the aerobic flora is slowly replaced by an anaerobic flora, the coliform bacteria generally falling to below 10^8 /g while the faecal enterococci decrease to below 10^7 /g. The anaerobic flora continues to develop and increase in complexity. By the end of the first week high numbers of clostridia and some nonsporing anaerobes can be isolated. At 2 weeks the anaerobic cocci (peptostreptococci) outnumbered all the other groups of bacteria, forming about 30% of the population, but as the birds grew older their numbers decreased. More than 40 different types of non sporing anaerobes have been isolated. At least 17 named species of clostridia and a number of unnamed strains have been isolated from chicken, turkeys, and pheasants.

From the microbiologist's standpoint, the intestine can be divided into three sections: the duodenum and small intestine, where the numbers of bacteria are relatively low, generally less than 10^8 /g intestinal content, the caeca, where a considerable microbial fermentation occurs, the number of bacteria present being approximately 10^{11}/g caecal content and the large intestine, which in most birds is relatively short and includes organisms from both the small intestine and caeca. Prior to hatching, the intestinal tract of the chicks is usually sterile and the intestinal flora is derived exclusively from the environment. Within a few hours of hatching, faecal enterococci, enterobacteria and sometimes clostridia, can be found multiplying in the caeca and scattered randomly throughout the rest of the alimentary tract. High levels of faecal enterococci and enterobacteria persist for several days in the duodenum and small intestine, the lactobacilli become established only by about the third day, but by the seventh day they have almost completely replaced the other bacteria. Faecal enterococci often found with the lactobacilli in the small intestine but very high numbers of coli- bacteria or clostridia are unusual. The caecal flora takes several weeks to develop. Although 10^{10} -10^{11} bacteria/g intestinal content, from the first day onwards the initial flora was shown to consist almost entirely of faecal enterococci and coli-bacteria with lactobacilli slowly increasing in numbers. After about the fourth day all three groups are gradually replaced by an anaerobic flora, their numbers falling to below 10^9 /g intestinal content. The caecal flora, which is predominantly composed of obligate anaerobes, continues to change for several weeks and shows increasing in the complexity (BARNES et al. 1979).

Development of the normal intestinal microflora of the small intestine, caecum and large intestine of SPF chicken, was studied in the period from hatching to 84 days of age. No bacteria were detectable at hatching, but by day 3, greater than 10^7 faecal enterococci and coliforms, 10^4 *Proteus* spp. and 10^5 clostridia /g of caecal content were present. By 7 days of age, these were accompanied by *Proteus* spp. at the levels in excess of 10^7 /g. These groups were the only facultative anaerobes found to colonize the caecum. Anaerobes appeared to colonize the caecum more slowly;

Eubacterium spp. did not appear before 10 days, *Bacteroides* spp. before 21 days or *Lactobacillus* spp. before 42 days. Other anaerobes (*Acidaminococcus* spp., *Peptostreptococcus* spp., *Propionibacterium* spp., and *Megasphaera* spp.) detected were transient, being detected only one or twice throughout the experimental period (COLOE et al. 1984).

The distributions of bacterial species within the chicken intestinal tract were as follow:
(A) Small intestine. The development of the flora of the small intestine was restricted to only four species. Initially faecal enterococci and coliforms colonized at day 3 at 10^2 and 10^3 /g respectively and by day 7 had each reached 10^7 /g. These organisms remained at 10^7 /g until day 42 when *G* spp. and *Eubacterium* spp. appeared at 10^7 and 10^8 /g respectively.
(B) Caecum. No bacteria were present at hatching (day 1), but by day 3 high concentration (10^8 /g) of faecal enterococci and coliforms (including *E. coli*) had developed and by day 7 there was also greater than 10^7 /g of *Proteus* spp. These three groups of bacteria were the only facultative anaerobes that colonized the caecum. The anaerobes were slower in colonizing the caecum and only small numbers of *Clostridium* spp. were established by day 3. Other anaerobes were then detectable at varying intervals, with *Eubacterium* spp. appearing at 10 days, *Bacteroides* spp. at 21 days, and *Lactobacillus* spp. at 24 days after hatching. Each of these species once established were regularly isolated as part of the normal flora.
(C) Large intestine. The composition of the bacterial flora of the large intestine closely resembled that of the caecum, but bacterial population densities were reduced by approximately ten-fold of each species. Only once was an anaerobe recoverable from the large intestine that was not also present in the caecum. The *Eubacterium* spp. isolated in this instance was, however, also present in the small intestine at that time (COLOE et al. 1984).
An analysis was made of the anaerobic bacteria isolated from 5-week-old chicken, the organisms being divided into groups based on some morphological criteria:
Gram-positive rods; three groups of organisms were differentiated by their characteristic morphology. The group 1 strains consisted of small rods, which tended to grow in irregular chains. The group 2 organisms were typically bifidobateria whilst the group 3 strains formed long chains of cells.
Gram-negative curved rods; these organisms which morphologically resembled spirilla were the most difficult to isolate and purify. No detailed studies have yet been made with them. The gram-negative anerobic bacteria comprise about 40 % of the total flora. The gram-positive non-sporing rods occur in almost equal numbers whilst peptostreptococci occur at about 15 % of the total flora. Other types of organisms notably curved rods were also present, but not enough strains to enable any assessment to be made of their real distribution in the caecum (BARNES and IMPEY 1970).

2.2 Functions of gastrointestinal tract

2.2.1. General features of digestion and absorption

There are four means by which digestive products are absorbed: active transport, passive diffusion, facilitated diffusion, and endocytosis. Active transport involves the movement of a substance across

the membrane of the absorbing cell against an electrical or chemical gradient. It is carrier-mediated, that is, the substance is temporarily bound to another substance that transports it across the cell membrane, where it is released. The process requires energy and is at risk of competitive inhibition by other substances, that is, other substances with a similar molecular structure can compete for the binding site on the carrier. Passive diffusion requires neither energy nor carrier; the substance merely passes along a simple concentration gradient from an area of high concentration of the substance to an area of low concentration, until a state of equilibrium exists on either side of the membrane. Facilitated diffusion also requires no energy, but it involves a carrier, or protein molecule located on the outside of the cell membrane that binds the substance and carries it into the cell. The carrier may be competitively inhibited. Endocytosis takes place when the material to be absorbed, on reaching the cell membrane, is enfolded into it. That part is then pinched off into the cell interior. This process is similar to phagocytosis. Absorption of all food by the small intestine occurs principally in the jejunum, however, the duodenum, although the shortest portion of the small intestine, has an extremely important role. The duodenum receives not only chyme saturated with gastric acid, but pancreatic and liver secretions as well. It is in the duodenum that the intestinal contents are rendered isotonic with the blood plasma, i.e., the pressures and volumes of the intestinal contents are the same as those of the blood plasma, so that the cells on either side of the barrier will neither gain nor lose water. The bicarbonate secreted by the pancreas neutralizes the acid secreted by the stomach. This brings the intestinal contents to the optimal pH, allowing the various enzymes to act on their substrates at peak efficiency. A number of important gastrointestinal hormones regulate gastric emptying, gastric secretion, pancreatic secretion, and contraction of the gallbladders. These hormones, along with neural impulses from the autonomic nervous system, provide for autoregulatory mechanisms for normal digestive processes. Most salts and minerals, as well as water, are readily absorbed from all portions of the small intestine. The sodium is absorbed by an active process, and the necessary metabolic energy is provided by the epithelial cells of the mucosa of the small intestine. Sodium is moved from the lumen of the intestine across the mucosa against a concentration gradient (i.e., a progressive increase in the concentration of sodium) and an electrochemical gradient (i.e., a gradual increase in the concentration of charged ions). Sodium ions are absorbed more readily from the jejunum than from other parts of the small intestine (STANOGIAS and BEARCE 1985).

Functions of the avian caecum; much has been written about the considerable variation in size and form that exists among avian caeca, and various theories have been advanced that relate the caecal size and function to the dietary habits and nutritional requirements of a particular bird. A few birds (e.g., parrots) have no trace of caeca, in other birds the caeca may be vestigial or rudimentary as in the case with hawks and falcons, and the closely related nocturnal owls have pronounced sacculate caeca. The greatest caecal development is found among gallinaceous birds in which the paired caeca may form a considerable part of the body weight. The caeca of Willow Ptarmigan were found to have an average length of 53 to 55 cm for each caecum (diameter only 0.7 cm) and were approximately 5 % of the total body weight. The browsing galliforms (grouse, ptarmigan and

capercaillie) have proportionately longer caeca than the grain-eating quails, partridges, pheasants, and turkeys. It has been suggested that the caeca may be:
A site for microbial digestion of cellulose.
A site for digestion of protein and carbohydrates.
A site of microbial synthesis and absorption of vitamins.
A site of absorption of nonprotein nitrogen.
A site of absorption of water.
Most of the studies carried out to explore these possibilities have been made with the gallinaceous birds. There is no evidence that any considerable digestion of cellulose occurs in the avian caecum. Occasionally cellulolytic bacteria have been isolated from chicken and pheasants in low numbers (BARNES and MEAD 1972, BARNES et al. 1972). They were not found in the caeca of the grass-eating goose, and there was also no evidence that any significant quantity of grass ever entered the caecum. The total time taken for the grass to pass through the goose was sometimes as little as 40 min but generally about 2 hr. A study was made for the ability of 20 different groups of nonsporing anaerobic bacteria, isolated mainly from the caeca of chicken and pheasants, to attack various substrates of possible ecological importance in the caecum The strains were found to be predominantly saccharolytic, only *Propionibacterium* acnes isolated from pheasants showed proteolytic activity. None of the strains hydrolysed amorphous cellulose or xylan. A few were weakly proteolytic, some hydrolysed starch. Further confirmation of the lack of proteolytic activity among chicken caecal anaerobes has been obtained by using differential casein medium; no significant numbers of proteolytic organisms were isolated. Thus the digestion of undegraded protein seems an unlikely function for the caecal flora (BARNES and IMPAY 1972).

2.2.2 Barrier function of GIT

2.2.2.1 Physiological and chemical barriers of GIT

2.2.2.1.1 Intestinal epithelium

The digestive tract is a tube lined by specialized epithelial cells that are continuously covered with the epithelial layers like the skin. Thus, the digestive tract is open to the external environment and to potential exposure to organisms and toxic agents that are introduced by ingestion. Along the length of the tract, the epithelial cells differentiate into a variety of cells with special functions that include the secretion of various fluids, electrolytes, enzymes, and in the gizzard, physical disruption of particulate digesta. The cells form a semipermiable surface that selectively allows passage of fluid, electrolytes, and dissolved nutrients. Regardless of its specialized function, every epithelial cell in the digestive tract is part of a continuous physical barrier to protect against the entry of foreign materials and organisms into the bloodstream and gaining access to other viscera. The integrity of this protective barrier is broken when organisms and toxic agents damage epithelial cells. This epithelial lining continually sheds cells into the center of the digestive tube (lumen) with ongoing regeneration of new cells that will differentiate to assume the functions of those lost.

Enterocytes provide nearly all of the lumenal exposure. Their structure and histochemistry in fowl are a direct parallel to all other animals. Goblet cells are minor in number by comparison, but they are important to overall intestine operation. PASTOR et al. (1988) reported two types of mucus producing cells. Most abundant are the ones producing sialo-mucins, whereas, a second type contains both sialo- and sulpho-mucins, and increases in number from duodenum to the ileo-caecal juncture. Microvilli greatly expand each enterocyte's surface exposure. Contractile elements provide convective movement, whereby immobilized enzymes, finalize digestion and improve the likelihood of absorption. In association with the microvilli are also arrays of N- and 0-linked glycoproteins that act as lectins and are speculated to have significance in parasite interactions.

In the avian gut, villi exist throughout the length of the small and large intestine, steadily decreasing in height along the way. The luminal surface of each villus is, in turn, increased by many microvilli to facilitate absorption on the surface of the cells. Each villus is lined with epithelial cells (enterocytes) that are differentiated according to location on the villus to absorb fluids and nutrients (tip), secrete electrolytes and fluids (side and crypt), and to regenerate and replace damaged cells or those lost to normal attrition (crypt) (ELSON 1997).

2.2.2.1.2 Mucus and fluid secretion

Mucin released from specialized epithelial cells arranged into glands in the mouth and esophagus, and by individual goblet cells in the proventriculus and intestine and from goblet cells entrapped within the glycocalyx-microvilli network to create an "unstirred water layer" at the surface (NIMMERFALL and ROSENTHALER 1980). This viscoelastic gel acts as a three dimensional screen which excludes all particulates and restricts diffusion of large molecular weight compounds. Mucus lubricates movement of digesta along the digestive tract. Mucus is not secreted in the crop or gizzard, however, digesta arrives in those organs softened and lubricated by the previous upstream site. Mucus is a viscous material composed of water and glycoprotein. It protects the mucosal cells in the stomach and intestine from autodigestion by gastric acid, pepsin and other digestive enzymes. The protective effect of mucus is further evidenced by increased secretion on the mucosal surface and goblet cell hypertrophy in response to noxious stimuli. Mucus is one of the barriers to bacterial and fungal invasion. Virulent strains of *Candida (C.) albicans*, the agent of thrush, have a mucinolytic enzyme that dissolves the mucin barrier to enhance adherence to and penetration of epithelial cells. In addition to mucus, the gut secretes large amounts of water mixed with electrolytes. It is estimated that for every gram of food ingested, the gut secretes about 2 grams of water that facilitates digestion and absorption. The excessive water in the lumen is reabsorbed in the lower small intestine, cecum, and colon. The fluid in the upper small intestine, however, is protective in that it keeps bacteria in suspension and washes them downstream (ELSON 1997).

The attachment of bacteria to mucosal surfaces is the initial event in the pathogenesis of most bacterial infections that originate at mucosal surfaces, such as the gut. The intestinal mucus layer appears to function as a defensive barrier limiting micro-organisms present in the intestinal lumen

from colonizing enterocytes. Protein malnutrition was not associated with bacterial translocation and measurement of enteroadherent, mucosally associated bacterial population levels documented that the total number of gram-negative enteric bacilli adherent to the ileum and caecum was less in the protein-malnourished rats than in the normally nourished animals. Furthermore, there was an inverse relation between the duration of protein malnutrition and bacterial adherence to the intestinal mucosa. In contrast, after endotoxin challenge, the level of enteroadherent bacteria was increased and bacterial translocation was observed. The binding of *E. coli* to immobilized ileal mucus in vitro was decreased significantly in protein-malnourished rats, whereas *E. coli* binding to insoluble ileal mucus was increased in the rats receiving endotoxin. This study indicates that the adherence of bacteria to the intestinal mucosal surface is an important factor in bacterial translocation, that intestinal mucus modulates bacterial adherence, and that increased levels of mucosally associated bacteria are associated with a loss intestinal barrier function to bacteria (KATAYAMA 1997).

2.2.2.1.3 The lamina propria

The epithelial lining of the gut is supported by the lamina propria, which contains the connective tissue that underlies the specialized surfaces, the vascular and lymphatic channels, and the immune system, or gut-associated lymphoid tissue (GALT). The blood vessels going into and away from the tip of the villus form a counter current mechanism that creates a hyperosmolar condition to facilitate absorption of fluid. Throughout the gut, the rich vascular supply serves to rapidly dilute and carry away any agents or chemicals (endogenous or exogenous) that may breach the mucosal barrier. Agents that directly damage the components of blood vessels can interfere with intestinal integrity by causing ischemic injury to the mucosa (infarction), or leakage of blood from the vascular channel (hemorrhage). Virulent forms of Newcastle disease and avian influenza, invasive candidiasis, coccidiosis caused by *Eimeria tenella*, and the more pathogenic forms of salmonellosis are examples of diseases that can injure the gut vascular system (ELSON 1997).

2.2.2.1.4 Enzymes, acids, and biochemical barriers of intestinal environment

The gastrointestinal tract has a series of effective barriers, including acid in the stomach and the antibacterial activity of pancreatic enzymes, bile, and intestinal secretions help in removal of harmful microorganisms.

2.2.2.1.4.1 Acidity of the stomach

The normal pH of the stomach is less than 4. This acidity spills into the small intestine establishing a pH gradient that prevents most bacteria from colonizing the stomach, duodenum, jejunum and upper half of the ileum. Because of this, the majority of ingested pathogens never reach the intestinal tract. Over 99.9% of ingested bacteria are killed after 30 minutes exposure to stomach acidity. Alteration of the acid barrier of the stomach by disease, drugs increases the survival of pathogens across this organ and may lead to microbial infection downstream. The areas of the gut

where microorganisms are found in high numbers usually have a pH between 5.0 and 7.5. The stomach of simple-stomached animals generally do not support microbial growth because the high acidity of the contents. However, in several species of animals, significant numbers of bacteria have been found in less acid regions of the stomach such as the anterior and non-glandular regions. Lactobacilli are the commonest bacteria found (CLARKE 1977). Hydrogen ion concentration is a major factor dictating what types of microbes can colonize habitats in the stomach and upper small intestine (SMITH 1965). The pH values of the caecum of conventional birds were always much lower in the presence of lactose. The three species of bacteria, which came closest to producing this effect, were *E. coli* and *L. salivarius* and *E. faecalis* (MORISHITA et al. 1982). The pH affects the antibacterial activity of the volatile fatty acids (VFAs) by altering the amount of dissociation of the acid. The undissociated lipophilic form of the the acid is responsible for the antibacterial activity of VFA because it can more easily penetrate bacteria cell walls. Once inside the cell, the acid may dissociate and kill the cell. The amount of the undissociated acid present is determined by the pH of its enviroment. The antibacterial activity of VFAs increases as the pH and Eh decrease (HINTON et al. 1990).

2.2.2.1.4.2 Bile acids

Bile acids are a group of related amphiphilic steroids, possessing both a hydrophilic and a hydrophobic face. The primary bile acids, cholic acid and chenodeoxycholic acid, are synthesized from cholesterol in the liver and released into the bile conjugated to glycine or taurine in order to solubilize fats and cholesterol for uptake in the small intestine. In the colon, bile acids that are not absorbed and recycled by the enterohepatic circulation are mostly deconjugated and 7-dehydroxylated to give the secondary bile acids, deoxycholic and lithocholic acid. Conjugation and the presence of hydroxyl groups give the primary bile acids more hydrophilic character. Bile solubilizes lipids; it thus inactivates those organisms having a lipid envelope. All enveloped viruses and many bacteria are thus prevented from growing in areas of high bile salts. The same bile acids, which are essential for digestion and absorption of dietary fats in the intestinal tract, as well as the metabolism of cholesterol, may play an important role in regulating the composition of the normal intestinal microflora by inhibiting growth of microbes not normally found in the intestines (BINDER et al. 1975, SAVAGE 1977). The in vitro growth of many different intestinal facultative and anaerobic bacteria is inhibited by low concentrations of unconjucated bile acids (BINDER et al. 1975, FLOCH et al. 1972). However, the majority of in vivo investigations have failed to demonstrate significant antibacterial activity of unconjucated bile acids (FLOCH et al. 1972).

2.2.2.1.4.3 Oxidation-reduction potential

Oxygen utilization by the microbial components of the intestinal normal flora produces anaerobic microenvironments. Both commensally and pathogenic anaerobes can flourish under these conditions, but the growth of pathogens requiring oxygen would be restricted (KOOPMAN et al.1975, LEE and GEMMELL 1975). For example lactobacilli change the oxidation-reduction

potential through its production of metabolites by making the environment less conductive for organisms requiring oxygen.

2.2.2.1.4.4 Hydrogen sulphide

Hydrogen sulphide restricts the range of substrates, which a given bacterial species can efficiently utilize under anaerobic conditions, and this may be a factor by which the normal flora inhibits the growth of exogenous microorganisms (FRETER et al. 1983).

2.2.2.1.4.5 Elaboration of antibiotic – like substances

The fourth general mechanism of direct bacterial antagonism is the production of antibiotic-like substance by one microorganism, which inhibits the multiplication of another. The chemical nature and mode of action of these inhibitory substances are quite diverse and include ammonia, hydrogen peroxide, hemolysins, lysostaphin, bacterial enzymes, bacteriophage tails, defective bacteriophage, and bacteriocins. Of the entire intestinal microflora of the avian intestinal tract, the *Lactobacillus* genus predominates similar to the condition found in the mammalian gastrointestinal tract. In the chicken there are three predominant species of *Lactobacillus* (*L.*) (*L.reuteri, L. salivarius, and L. animalis*), but only *L. reuteri* has the potential to produce reuterin, a clearly defined antibacterial substance that is an intermediary metabolite of glycerol (TALARICO et al. 1988). *L. reuteri* was first isolated by Lerche and Reuter at 1980, but until 1983 was classified as *L. fermentum*. Reuterin, has broad-spectrum antimicrobial activity extending to the inhibition of at least 25 different genera of prokaryotic microbial pathogens (both gram-negative and gram-positive) and at least 10 different eukaryotic protozoan pathogens frequently found in intestines of most mammalian and avian species. *L. reuteri* cells normally reside the gastrointestinal tract of healthy chicken, with the highest numbers found in the crop and caeca. The presence of lactose (whey) increases the number of *L. reuteri* found in the caeca, and dietary lactose supplements have been reported to reduce the numbers of *Salmonella* found in gastrointestinal tract of chicken and turkeys (EDENS et al. 1997). Strains of *L. acidophilus* and other lactic bacteria have been found to possess marked inhibitory properties against intestinal pathogens as well as food spoilage organisms. Selective inhibition has been attributed in part to the ability of lactic acid organisms to produce natural antibiotics. *L. acidophilus* specifically has been reported as producing antibiotics, notably acidophillin, lactolin and acidolin. The acidolin production together with the lactic acid production is responsible for the excellent anti-microbial activity against enteropathogenic organisms including *E. coli, Klebsiella*, *Salmonella* spp., *S. aureus, C. perfringens*, *Pseudomonas aeruginosa* (green diarrhoea), and *Shigella* spp. The most extensively studied of the antibiotic-like compounds are the bacteriocins (IGLEWSKI and GERHARDT 1978). Practically all genera of bacteria have been shown to produce bacteriocins or bacteriocin-like compounds. A bacteriocin is defined as a diffusible substance produced by a microorganism, which possesses an essential biologically active protein moiety and has a bactericidal mode of action against other bacterial strains but not against the producing microorganism. The significance of bacteriocins as regulators of bacterial populations is unclear (BOOTH et al. 1977). Early investigators assigned a major role to bacteriocins or

bacteriocin-like substances in the regulation of microbial population. These investigators suggested that the stability of the intestinal flora and resistance to colonization by exogenous bacteria was due to elaboration of bacteriocins by the resident bacteria. Even though production of bacteriocins by enteric bacteria has been frequently demonstrated in vitro, evidence accumulated in recent years have failed to provide consistent evidence for an important ecological role of bacteriocins in the gastrointestinal tract (IKARI et al. 1969).

2.2.2.2 Bacterial barrier

The animal host and its intestinal microbial flora function together as a complex ecologic system in which there is a significant impact of the intestinal flora on the host as well as of components of the microbial flora on one another. Aerobic and anaerobic bacteria of the intestinal flora influence numerous anatomic, physiologic, and immunologic parameters of the host. Constituents of the indigenous intestinal flora also engage in a multitude of antagonistic and cooperative interactions. The normal bacterial intestinal flora represents an extremely important defense mechanism, which effectively interferes with the establishment of many important enteric pathogens. Mechanisms by which microorganisms suppress the growth of other microorganisms include modification of bile acids, stimulation of peristalsis, induction of immunologic responses, depletion of essential substrates from the environment, competition for attachment sites, creation of restrictive physiologic environments, and elaboration of antibiotic-like substances. Components of the intestinal microbial flora also interact synergistically in the induction of disease or the utilization of substrate (ROLFE 1984).

2.2.2.3 Immunological barrier (BUTCHER and MILES 1991)

2.2.2.3.1 Non-specific immune mechanisms

2.2.2.3.1.1 Genetic factors

Some strains of birds are genetically resistant to some diseases like leukosis.

2.2.2.3.1.2 Body temperature

The high body temperature of the chicken gives protection against many diseases.

2.2.2.3.1.3 Anatomic features

Many disease organisms cannot penetrate intact body coverings like feather, skin and the epithelial lining of the digestive systems. They perform the first line of defences against harmful agents and pathogenic bacteria. Some nutritional deficiencies or infectious diseases affect the integrity of the body coverings, allowing penetration of disease organisms.

2.2.2.3.1.4 Normal microflora

The microflora present in skin and GIT gives protection against the harmful bacteria.

2.2.2.3.1.5 Peristalsis

The continuous unidirectional flow of material through the lumen of the upper and middle regions of the small intestine is a strong influence preventing microbial communities from developing unless they can attach to underlying epithelial structures (SAVAGE 1977). Motility facilitates nutrient absorption by maintaining a maximum concentration differential between luminal contents and the unstirred water layer. A well developed nervous system (AISA et al. 1990) coordinates bolus progression to balance energy expenditure with nutrient recovery. Fowl employs a to and fro refluxing as a means of convection, which lends to a prominent circular muscle (TURK 1982). Such refluxing is particularly active between the gizzard and distal duodenum where bile and pancreatic juice enter (CHAWAN et al. 1978, OGURO and IKEDA 1972, SKLAN et al. 1978). High environmental temperature reduces the extent of motility and rate of nutrient uptake, as do circumstances that increase viscosity of luminal contents (VAN DER KLIS et al. 1993). Objective of the large intestine is to salvage nutrients remaining with indigesta from the small intestine together with those excreted in the urine. Essentially, a coordinated effort of motility, microflora, and mucosa recovers water, sodium, and volatile fatty acids. Motility coordination is central to function. Indigesta from the ileum are ejected into the colon through the ilea-colonic sphincter. Sphincter opens and closes rapidly to maintain a low microbial level in the small intestine where operations favor aerobic conditions, as compared to the large intestine where a dense population depends on an anaerobic environment. Gentle retroperistalsis starting in the urodeum transfers urine and fine particulates through small orifices into the caeca. Coarse particulates segregate to the colon core and progress to the coprodeum where a critical mass accrues for defecation (DUKE 1989).

2.2.2.3.1.6 The phagocytic cells

There are two primary phagocytic cell types, the granulocytes, which include the neutrophils and eosinophils, and the mononuclear phagocytes, which include monocytes circulating in the blood and macrophages located in tissue. Phagocytic cells are responsible for engulfing, killing and digesting invading bacteria, they also have an important role in controlling viral infections. Phagocytic cells are attracted to infected or inflamed sites by chemotactic substances which may be produced by certain microorganisms, be generated by cleavage of certain complement components, or be released by sensitized lymphocytes. As the phagocytes arrive at the infected area, they begin to engulf the infectious agent if it is susceptible to phagocytic activity. Most disease-causing microorganisms must be opsonized before they can be engulfed by phagocytes, bacteria are opsonized by the attachment of a specific antibody and/or complement to their surface. This in turn allows for easier destruction of the bacteria by phagocytic cells. Enzymes within the phagocytic cell will attempt to destroy the bacterium after they have been engulfed. Phagocytes may also play an important part in controlling certain viral infections. Since it is necessary that viruses be within a

will attempt to destroy the bacterium after they have been engulfed. Phagocytes may also play an important part in controlling certain viral infections. Since it is necessary that viruses be within a cell (intracellular) to replicate and thus cause disease, the phagocyte will attempt to destroy the infected target cell if it has been properly tagged by a specific antibody produced by the acquired immune system. Even though the mechanism of destruction is not completely understood, the antibody presumably creates a bridge between the phagocyte and the infected target cell; the phagocyte will then attempt to destroy the infected target cell and thus destroy the virus. All phagocytic cell types are capable of the activities described above, however, the granulocyte and most specifically the neutrophil is the most proficient of all the phagocytes in these activities. Mononuclear phagocytes originate in the bone marrow as monocytes and are released into the blood stream where they circulate before migrating into the tissues to become macrophages. Even though macrophages are capable of all the activities of the neutrophil, macrophages are described as the second line of defense. They are not as aggressive as the neutrophil and are much slower to arrive at the sites of infection. Macrophages, however, are capable of much more sustained activity than neutrophils and are able to kill certain types of bacteria that are resistant to killing by the neutrophils. In addition, macrophages process engulfed microorganisms and present fragments (antigens) of them to specialized cells for initiating the antibody-mediated (humoral) immune response and the cell-mediated immune response to infectious diseases agents (JEURISSEN et al. 2000).

2.2.2.3.1.7 The complement system

The complement system consists of at least 20 serum proteins which, when stimulated, respond with a series of chain reactions similar to the "domino effect," i.e., something happens to activate the first component which in turn activates the next component which in turn activates the next component, etc., until the reaction is complete. The primary effect of this chain reaction is to increase blood flow to an injured area of the body or an area that has been invaded by an infectious agent, the increased blood flow to the area is accompanied by fluid loss from the blood vessels. This complement system reaction, which at times appears to be detrimental, hastens the accumulation of disease fighting cells (phagocytes) to the area by providing a fluid "pathway" and by producing chemotactic substances, which attract phagocytes. Components of the complement system may also be able to lyse (rupture) the microorganism or opsonize (tag) the infectious agent, which renders the infectious agent more vulnerable to a phagocytic cell (ROTHER 1977).

2.2.2.3.1.8 The interferon system

Interferons are small proteins that cells immediately secrete when they are invaded by infectious agents, especially viruses. Interferon controls replication of certain viruses by inhibiting production of required viral protein in the infected cells and signals other body cells to initiate defenses which prevent replication of viruses in cells if they should be attacked. Interferon further enhances the immune system by increasing the activity of phagocytes in the destruction of engulfed microorganisms and engulfed infected cells. Interferons are not viral- specific, that is, interferon

will cross-protect against a variety of viruses. In addition, interferon levels secreted by invaded cells can reach protective levels very quickly, thus preventing replication of viruses in other cells (LOMNICZI 1970).

2.2.2.3.2 Specific immune mechanisms

There are many similarities between the general immune mechanisms of mammals and chickens.

2.2.2.3.2.1 The non-celluar component

There are three principal classes of antibodies in birds i.e., IgM, IgG (also called IgY), and IgA. IgM appear after 4-5 days following exposure to a disease organism and then disappear by 10-12 days. IgG is detected after 5 days following exposure, peaks at 3 to 3 1/2 weeks, and then slowly decreases. IgG is the important protective antibody in the chicken and is measured by most serological test Systems. IgA appears after 5 days following exposure. The cells that produce antibodies are called B-lymphocytes. These cells are produced in the embryonic liver, yolk sac and bone marrow bursa of Fabricious (BF). The BF programs these cells, which then move to the blood, spleen, caecal tonsils, bone marrow, Harderian gland, and thymus. Chickens have well developed secondary lymphoid structures. These include spleen, Harderian gland, bone marrow, gut-associated lymphoid tissue, conjunctival-associated lymphoid tissue, head-associated lymphoid tissue, and bronchial associated lymphoid tissue. Chickens lack functional lymph nodes, lymphatics with associated lymphoid nodules. In addition, many organs such as liver, kidney and pancreas have diffuse lymphoid cells scattered in the parenchyma. Destruction of the BF at a young age by some viral diseases like Gumboro disease or Marek's disease prevents programming of B-cells. Thus, the chicken will not be able to respond to diseases or vaccinations by producing antibodies. The macrophage engulfs a disease organism when enters the body and transports the disease organism and exposes it to the B-lymphocytes. The B-cells respond by producing antibodies after day 5 following exposure. If the chicken is exposed a second time to the same disease, the response is quicker and a much higher level of antibody production occurs. Antibodies perform their function by attaching to disease organisms and blocking their receptors. The disease organisms are then prevented from attaching to their target cell receptors in the chicken. The attached antibodies also immobilize the disease organism, which facilitates their destruction by macrophages (BUTCHER and MILES 1991, SHARMA 1997).

2.2.2.3.2.2 The cellular component

Includes all the cells that react with specificity to antigens, except those associated with antibody production. The cells associated with this system, the T-lymphocytes, begin as the same stem cells as the B-cells. However, the T-lymphocytes are programmed in the thymus rather than the BF. The T-lymphocytes include a more heterogeneous population than the B-cells. Some T-cells act by producing lymphokines (over 90 different ones have been identified), others directiy destroy disease organisms, some T-cells act to enhance the response of B-cells, macrophages, or other T-cells (helpers), and others inhibit the activity of these cells (suppressors). The cellular system was

identified when it was shown that chicken with damaged BF could still respond to and eliminates many disease organisms (SHARMA 1997).

2.2.2.3.3.1 The gut-associated immune system (GALT)

The gastrointestinal mucosa separates the intraluminal gastrointestinal fluid, which contains a high number of antigens from different sources, and prevents free access of antigens to the body. Simultaneously, it allows some vital host-environment interactions. A number of unspecific factors are important in preventing antigen invasion. The specific mucosal immunity is related to secretory IgA. IgA is derived from mucosal plasma cells after antigen-induced proliferation of its precursors in Peyer's patches. These IgA-positive B-lymphoblasts migrate through the systemic circulation and then "home" to the mucosa. IgA is translocated as a dimer to the gut lumen after attachment to the secretory component (SC). Part of it is excreted into the bile via small bile ducts after portal and possibly systemic circulation and binding to SC. T cells and mast cells are also considered to show migration and homing phenomena. In addition to the gut, some other mucosa-associated lymphoid tissues, (e.g. bronchus, mammary, salivary and lacrimal glands as well as the female genital tract), can participate in homing. Little is known about the local regulatory mechanisms, which allow an immunoglobulin class specifity of immune responses. Induction of local immunity and specific systemic tolerance seems to be a characteristic immune response of the GALT (BORSCH 1984).

The intestine is the largest immunological organ in the body. It comprises 70-80 of all immunoglobulin-producing cells and produces more secretory IgA (sIgA) (50-100 mg/kg body weight/day) than the total production of IgG in the body (ca. 30 mg/kg/day). The local immune system of the gut has two main functions: to protect against enteric infections, and to protect against uptake of and/or harmful immune response to undergraded food antigens. The best known entity providing specific immune protection for the gut is the sIgA system. The resistance of sIgA against normal intestinal proteases makes antibodies of this isotype uniquely well suited to protect the intestinal mucosal surface. The main protective function of sIgA antibodies is the "immune exclusion" of bacterial and viral pathogens, bacterial toxins and other potentially harmful molecules. SIgA has also been described to mediate antibody-dependent T cell-mediated cytotoxicity (ADCC), and to interfere with the utilization of necessary growth factors for bacterial pathogens in the intestinal environment, such as iron. It is now almost axiomatic that in order to be efficacious, vaccines against enteric infection must be able to stimulate the local gut mucosal immune system, and that this goal is usually better achieved by administering the vaccines by the oral route rather than parenterally. Based on the concept of a common mucosal immune system through which activated lymphocytes from the gut can disseminate immunity also to other mucosal and glandular tissues (MCGHEE and KIYONO 1993).

2.2.2.3.3.2 Interactions between the intestinal microflora and the GALT

After birth, the intestine is rapidly colonized by bacteria, which probably act as a source of antigens and non-specific immunomodulators. The dual role of the digestive flora on the immune System should be emphasized. Bacteria can be considered as antigens able to elicit specific systemic and local immune responses. Furthermore, they exert a considerable influence on the number and distribution of the GALT cell populations and play an important role in the regulation of immune responses. The cellular and molecular events by which the digestive fiora modulates the immune System are still poorly understood. The digestive flora is the major antigenic Stimulus responsible for the migratory pathway and maturation of precursor lymphoid cells present in the Peyer's patches. Consequentiy, it acts on the development and maturation of the IgA plasmocytes. In germ-free mice, IgA-plasmocyte number is decreased tenfold as compared with controls. 1t has been shown that the sequential establishment of the digestive flora from birth to weaning is responsible for the progressive increase in IgA plasmocyte numbers in the lamina propria of the small intestine in the growing normal mouse. Gram-negative bacteria such as *E. coli* and *Bacteroides* play an important role in this immunologically non-specific effect (PABST 1987, ELSON 1985). The digestive flora also modulates the specific immune responses at local and systemic levels. 1t allows the persistence of the systemic unresponsiveness to an antigen, induced by a previous feeding with the same antigen (oral tolerance), and shortens the abrogation of oral tolerance mediated by cholera toxin or *E. coli* toxin, which seems to be a property of gram-negative bacteria (MOREAU and GABORIAU-ROUTHIAU 1996).

2.3 Effect of the age on the gastrointestinal flora

SMITH (1965) and ZIPRIN et al. (1989) have reported that the newly hatched chick is very susceptible to *Salmonella* infections, but as the chick ages through 5 day posthatch its resistance increases. They concluded that increasing resistance to *Salmonella* infections could be attributed to development of a competent T cell-dependent immune system, however, this did not rule out the contributing effects of acquired resistance through colonization of the intestinal tract of beneficial microflora. In fact the increasing resistance to *Salmonella* infections after hatch may well be dependent upon both the development of the T cell-mediated immune response and the acquisition of beneficial microflora. The timing of these two independent events would appear to be more than a simple coincidence.

GAST and BEARD (1989) found that chicks inoculated orally between 1 and 8 day posthatch with S. Typhimurium showed decreasing mortality with increasing age, but the pathogenic organism persisted in the caeca of the 7-week-old broilers. They also noted that the birds inoculated at 1 day posthatch had greater numbers of *S.* Typhimurium cfu at 7 week than did the birds inoculated at 8 day posthatch. Furthermore, *S.* Typhimurium adhered to the epithelium of the caeca in the birds inoculated at 1 day more readily than in the birds inoculated at 3, 5, or 7 day posthatch, but age did not affect the recovery of the organism from the spleen or the liver.

2.4 Effect of the diet on the gastrointestinal flora

2.4.1 Influence of dietary carbohydrates on the gut flora of chicken

Some grains are known to contain naturally occurring substances that are harmful to the avian species. For example, barely and rye contains non-starch polysacchrides (pentosane, β-glucane and pectines) which have been shown to interfere with digestion by birds. A significant increase in mortality was observed in broilers fed wheat in crumbled diets. This increase in mortality was associated with necrotic enteritis with *C. perfringens* indicated as the causative pathogen and complicated by a coccidiosis outbreak. When yellow corn was used in the diet, mortality was 2.9%. Use of all wheat, ground with a hammer mill, increased mortality to 28.9%. However, roller mill-ground wheat diet resulted in a mortality of 18.1%. When the grain component was approximately 50% wheat and 50% corn, mortality was 12.6% for broilers fed hammer mill-ground wheat and 3.4% for roller mill-ground wheat. Grain and feed were tested for several mycotoxins. Low levels of deoxynivalenol were found in both corn and wheat diets, but no differences between the corn and wheat-based diets were found that would explain the incidence of enteritis (BRANTON et al. 1987).

Commercial broiler chicks were given a three-strain composite of bacitracin-resistant *C. perfringens* by oral gavage and were sampled periodically to determine the dynamics of *C. perfringens* colonization of the intestinal tract of broiler chicken and faecal shedding. After gavage, the chicks were divided into two groups and placed in isolators, one group received a traditional corn-based diet, and the other group received the same diet supplemented with 50% rye to place the birds under dietary stress. The numbers of bacitracin-resistant *C. perfringens* in various parts of the intestinal tract, liver, and faeces were determined using a selective plating medium containing bacitracin. In chicken on the corn-based or 50% rye diet, *C. perfringens* was isolated infrequently from the various parts of the intestinal tract, liver, or faecal droppings during the first 36 h following the last gavage. Addition of the rye to the diet increases the numbers of *C. perfringens* from 2 to 21 day of age in the ileum, caeca and faeces of birds given a diet containing 50% rye. Also, a higher frequency of recovery through most of the intestinal tract and higher numbers in the jejunum, ileum, and caeca of *C. perfringens*-positive birds occurred as compared to corresponding numbers in birds on a corn-based diet. Birds on the rye-based feed were observed to have decreased body weights compared to birds on the corn-based diet, but increased mortality was not observed from the use of rye-supplemented feed (CRAVEN 2000).

TAKEDA et al. (1995) conducted five experiments to determine the effects of dietary lactose and rye on caecal colonization of *C. perfringens* in white leghorn chicken. They found that, six days after oral inoculation of the organism, the numbers of *C. perfringens* organisms in the caecal contents were significantly lower in chicken on 2% and 10% lactose-supplemented feed than in chicken on unsupplemented feed. When *C. perfringens* was given in drinking water, 10% lactose supplementation was needed to significantly reduce the counts of *C. perfringens* 4, 6, and 8 days after feeding began. Effect of rye-ration on caecal colonization of *C. perfringens* was also examined. Counts of *C. perfringens* in caecal contents of chicken fed a diet containing 50% rye

were significantly higher than control values 4, 6, and 8 days after feeding began. When chicks were fed a diet containing both 10% lactose and 50% rye, *C. perfringens* counts in caecal contents were lower than in chicken fed 50% rye only at 6 days after feeding began. Results led to the conclusion that dietary lactose is effective in reducing the caecal colonization of *C. perfringens*.

Two-day-old chicks were orally inoculated with 1 ml of *S.* Typhimurium (10^5) colony-forming units/ml) and divided into four groups. Three groups were fed 2.5% carbohydrates starting on day 1 (arabinose, galactose, and lactose), while the fourth group served as the control. Caeca were obtained from each group at 7, 14, and 21 days. At the end of 14 days, all three carbohydrates statistically reduced *Salmonella* recovery. However, lactose failed to reduce recovery between day 14 and day 21. Arabinose and galactose continued to show significant reductions of recovery through 21 days, it appears that complete elmination of salmonellae from intestinal tract of chicks may require additional types of treatments. The physiological interaction between the intestinal tract flora and the carbohydrate added may extend beyond receptor site blockage and enter the area of reduction of the target organism by other means (MCHAN et al. 1991).

OYOFO et al. (1989a, b and c) evaluated the effect of carbohydrates in the drinking water of broiler chicken on *S.* Typhimurium colonization. They compared with dextrose, maltose, sucrose, lactose and mannose. He found that mannose and lactose (2.5%) significantly reduced the intestinal colonization of *S.* Typhimurium by at least one-half, as compared with dextrose, maltose, and sucrose. Lactose and mannose also significantly reduced the mean $\log_{10}$ number of *S.* Typhimurium in the caecal contents. They suggested that mannose might act through one or more mechanisms. Mannose blocks the adherence of bacteria that have mannose-sensitive fimbriae. It may act by binding to receptor sites on epithelial cells or by interacting with mannose-sensitive type 1 fimbriae on the bacteria. Lactose however may act by a completely different mechanism. Lactose probably does not offer protection against *S.* Typhimurium by promoting the growth of lactobacillae alone. It is likely that lactose also promotes the growth of lactose fermenting bacteria that either compete with *S.* Typhimurium for colonization sites or produce volatile fatty acids toxic to *S.* Typhimurium. Although mannose was the most effective sugar at blocking colonization, lactose may be more practical because it is effective and costs much less than mannose. Provision of carbohydrates in the drinking water had no significant effect on weight gain. In the intestinal tract of weaned animals lactose is less readily digested than other sugars such as glucose, galactose, sucrose and maltose.

Since chickens have only a trace of lactase activity in their intestinal tissues (SIDDONS and COATES 1972) lactose enters the large intestine in much higher concentrations than do other sugars and, therefore, it is more effective in producing changes in the flora of organ. Many workers have reported the effects of dietary lactose on the gut flora of chicken. One consistent finding in these studies was that the inclusion of lactose in diet increased the numbers of lactobacilli or bifidobacteria in the gut, while the effects on other groups of bacteria were more variable. MORISHITA et al. (1982) compared the gut microflora of chicks fed on a purified diet containing

300 g lactose plus 300 g starch / kg with that of control birds receiving a diet containing 600 g starch / kg. *E. coli* was consistently present throughout the intestine and was unaffected by the presence of lactose in the diet. The only group which showed a marked and consistent response to lactose was *Proteus* spp. which was present in large numbers in the caeca of control birds but not detectable in the gut of birds receiving lactose. The presence of lactose had a suppressing effect on *Proteus mirabilis* when grown in the presence of other bacteria, like *E. coli* and *E. faecalis* but not when grown alone. Conversely, *L. acidophilus* was suppressed by lactose when in pure culture but not when grown in the presence of other bacteria. The enterococcus flora contained *E. faecalis* either as the predominant components or as the sole species present. *E. faecalis* was suppressed by the presence of other bacteria. This may also due to inhibition by lactobacilli or other more complex microbial interactions.

Lactose did not affect the count of *E. faecalis* or the total enterococci count. Lactobacilli were commonly isolated from the gut and lactose had the effect of reducing the incidence of *L. acidophilus* and *L. salivarius* in the caecum at 14 day although *L. salivarius* was present its numbers exceeded those found in the control chicks. Bifidobacteria were only isolated in small numbers from the birds receiving lactose. The occurrence of *Bacteriodes* was also variable but there was a tendency for the counts to be lower in the caecum of 14-day-old birds fed on lactose. Clostridia were invariably present in large numbers in the caecum of control chicks but were found less frequently in the chicks fed on the lactose diet. The effect of dietary lactose and anaerobic cultures of caecal microflora of mature chicken on the colonization of young broiler chicken by *S.* Typhimurium was evaluated. Newly hatched chicks were given no treatment (controls), anaerobic caecal cultures, lactose (2.5%) in the drinking water, or both anaerobic cultures and lactose. Chicks were challenged per os at 3 days of age with either (10^6) or (10^8) *S.* Typhimurium resistant to nalidixic acid and novobiocin. On day 10, the caecal contents of the chicks were examined for *S.* Typhimurium, pH, short-chained volatile fatty acids (VFAs), undissociated VFAs, and lactic acid. Chicks given either lactose alone or caecal anaerobes alone had significantly fewer *S.* Typhimurium recovered from their caeca than the controls. Chicks given the combination of dietary lactose and caecal anaerobes had significantly fewer *S.* Typhimurium recovered from their caeca than the chicks given dietary lactose or caecal anaerobes alone. During storage, anaerobic cultures from mature chicken's caecal material used in classical CE experiments have been reported to lose the ability to reduce *Salmonella* colonization of young chicks. Chicks given lactose had significant increases in the lactic acid concentration of their caecal contents. Increased lactic acid concentrations were directly correlated to decreased caecal pH values and caused a reduction in the total concentration of VFAs but a significant increase in the undissociated form of some VFAs (HINTON et al. 1990, HINTON et al. 1991).

The effect of oral inoculation with anaerobic cultures of caecal microflora and providing lactose in the feed on colonization resistance to invasive *S.* Enteritidis was evaluated in newly hatched leghorn chicks. *Salmonella* colonization of the caeca, tissue invasion and organ colonization, horizontal transmission, and seroconversion were significantly decreased in chicks inoculated with caecal

flora. The addition of lactose to the feed, in the absence of caecal microflora, failed to provide protection. Dietary lactose enhanced colonization resistance in chicks that were inoculated with anaerobic cultures of caecal flora. The results indicated that establishment of normal caecal flora in layer chicks together with the addition of lactose to the diet markedly increases resistance to caecal colonization and organ invasion, and decreases horizontal transmission of *S.* Enteritidis (CORRIER et al. 1991). The combined effect of treatments with dietary lactose plus anaerobic organisms on caecal colonization of broiler chicks by *S.* Typhimurium was evaluated. Chickens treated with a combination of anaerobic organisms and 7% dietary lactose were resistant to caecal colonization by *S.* Typhimurium. The number of recoverable *S.* Typhimurium cells per gram of caecal contents taken on days 10 and 15 after infection was significantly reduced. Treatment with anaerobes without the addition of lactose did not effectively control caecal colonization. Intracloacal inoculations with bacterial concentrations that varied by 10,000-fold resulted in roughly similar levels of colonization. The treatments resulted in reduced caecal pH and elevated levels of undissociated volatile fatty acids (ZIPRIN et al. 1990). The effect of providing lactose in feed and inoculation with volatile fatty acid-producing anaerobic cultures (AC) of caecal flora on *S.* Typhimurium colonization was evaluated in broilers. One-day-old chicks were divided into four groups and provided 1) no lactose, no AC, 2) AC, no lactose, 3) AC and lactose on days 1-10, or 4) AC and lactose on days 1-40. All groups were challenged per os with 10^6 *Salmonella* on day 3 and with 10^8 *Salmonella* on day 33. *Salmonella* growth in the caecal contents was significantly decreased on day 10 in the chicks provided lactose from day 1-10. However, after the removal of lactose from the diet, the chicks were susceptible to *Salmonella* colonization. The number of *Salmonella* in the caeca was significantly reduced in the chicks provided lactose throughout the 40-day growing period. Dietary lactose decreased the pH of the caecal contents and was accompanied by marked increases in the concentrations of undissociated bacteriostatic volatile fatty acids in the caecal contents. There is no significant difference on day 40 in the mean body weight of the chicken provided dietary lactose throughout the 40-day growing period and the groups of chicken not provided lactose. The data further suggest that dietary lactose inhibited *S.* Typhimurium growth by effectively enhancing natural host mechanisms of colonization resistance. The result suggest that supplementing poultry diets with low-cost lactose products may provide a means of significantly decreasing the number of *Salmonella* in the caecal contents of growing broiler chicks and reducing the number of market age broilers colonized by *Salmonella* (CORRIER et al. 1990). The effect of dietary lactose and *L. acidophilus* on the colonization of *S.* Enteritidis (phage type 4) in newly hatched leghorn chicks concurrently infected with *Eimeria tenella* was studied. There were six groups of birds in each of two replicate trials: uninfected controls, birds infected with *S.* Enteritidis, birds infected with *S.* Enteritidis and *E. tenella*, birds infected with *S.* Enteritidis and *E. tenella* that were fed with dietary lactose (5%), birds infected with *S.* Enteritidis and *E. tenella* that were provided with *L. acidophilus*, and birds infected with *S*. Enteritidis and *Eimeria* (*E.*) *tenella* that were fed both *L. acidophilus* and dietary lactose (5%). Chickens were necropsied at 2, 5, and 8 days after *S.* Enteritidis inoculation for bacteriological examination. The caecal population of *S.* Enteritidis was significantly increased by infection with *E. tenella.* Lactose alone and the

combination of lactose and *L. acidophilus* significantly reduced the population of *S.* Enteritidis in the caeca of birds infected with *E.* tenella, but the combination was more effective than lactose alone. Adding anaerobic culture of *L. acidophilus* alone did not significantly decrease the population of *S.* Enteritidis in the caeca. No significant reduction of colonization of *S.* Enteritidis in the liver and spleen of birds with coccidiosis was observed (QIN et al. 1995). Whey (5%) in the feed of chicks for the first 10 days of life reduced the mean $\log_{10}$ number of viable *S.* Typhimurium from 5.68 in control chicken to 3.38 in whey-fed chicken. Lactose in drinking water or reconstituted dry milk (5% wt: vol) in drinking water reduced the mean $\log_{10}$ number of *S.* Typhimurium to 2.60 and 2.11, respectively. Milk (5%) in feed was not effective in reducing *S.* Typhimurium colonization. The lack of effect of milk in the feed is believed to be because not enough lactose was provided at the 5% concentration. Lactose in whey or non-fat dried milk offers alternatives to the use of pure lactose in preventing or lowering *S.* Typhimurium numbers in young broiler chicken (DELOACH et al. 1990). The effect of 14 or 19 days of 10% dietary lactose administration on *S.* Enteritidis colonization and histological, morphometric, and organic acid changes of the caeca were investigated. At day 13 or 18, chicks were challenged with (10^8) cfu of *S.* Enteritidis. Chicks were killed and cultured 24 h later. A reduction in the total number of positive *S.* Enteritidis organ invasions was observed following 14 days or 19 days of treatment in chicks fed with lactose. Histological examination revealed a marked reduction in lamina propria thickness of caeca, as well as subjective epithelial cell proliferation from chicks following either 14 or 19 days of lactose administration. Using morphometric analysis, a reduction in the mean lamina propria thickness in chicken fed with lactose during 14 or 19 days was observed as compared with controls. Yet, an increase in the mean epithelial cell length in both lactose-treated groups was observed as compared with controls. Lactose decreased luminal pH and increased the concentration of acetic, propionic, butyric, and lactic acid. These data indicate that lactose-induced resistance to *S.* Enteritidis organ invasion is associated not only with an increase in organic acid concentration but also with measurable morphological changes of the caecal mucosa (TELLEZ et al. 1993).

The effect of dietary administration of lactose and poultry litter, containing caecal and faecal droppings from adult broilers, was evaluated for protective effects against *S.* Enteritidis colonization in leghorn chicks and 16-week-old hens. The addition of used litter as 5% of the feed ration significantly decreased *Salmonella* caecal and organ colonization in the chicks. Provision of used litter or used litter and lactose in the feed failed to provide protection against *Salmonella* colonization in the hens. The results indicated that resistance to *S.* Enteritidis colonization might be effectively increased in leghorn chicks by exposure to adult intestinal flora present in used litter. Furthermore, the results suggest that microbiological strategies employing adult intestinal microflora that increase *Salmonella* colonization resistance in young chicks may be ineffective in older hens (CORRIER et al. 1993).

Two-day-old chicks were orally inoculated with 1 ml of *S.* Typhimurium (10^5) colony-forming units/ml) and divided into four groups. Three groups were fed 2.5% carbohydrates starting on day 1

(arabinose, galactose, and lactose), while the fourth group served as the control. Caeca were obtained from each group at 7, 14, and 21 days. At the end of 14 days, all three carbohydrates statistically reduced *Salmonella* recovery. However, lactose failed to reduce recovery between day 14 and day 21. Arabinose and galactose continued to show significant reductions of recovery through 21 days. No statistical difference was found between *Salmonella* recovery from whole caeca (with caecal material) and inverted caeca (washed free of caecal material) (MCHAN et al. 1991). *Salmonella* contamination of the chicken's crop has been reported to increase markedly and significantly during feed withdrawal. Increasing pH during feed withdrawal has been attributed to decreased *Lactobacillus* fermentation within the crop and is associated with increased *Salmonella* recovery following feed withdrawal. As provision of lactose in water might serve as a fermentation substrate in crops, thereby causing a decreased crop pH. Provision of 2.5% lactose in the drinking water for 5 days (including the feed withdrawal period) caused a small but significant reduction of *Salmonella* recovery from crops. Extending the lactose exposure period to 11 days and reducing the feed withdrawal period to 12 hour did not improve the ability of lactose to reduce *Salmonella* recovery from crops or caeca. Previous studies demonstrating protective effects of lactose administration in the drinking water on *Salmonella* colonisation of the caeca were most effective when lactose was provided prior to *Salmonella* challenge. These data suggest that provision of 2.5% lactose in the drinking water during the last 5 or 11 days growout prior to slaughter will not be useful in an integrated *Salmonella* control program under commercial conditions (BARNHART et al. 1999). RIDDELL and KONG (1992) found that mortality due to necrotic enteritis was higher among chicken fed rations based on wheat, rye, barley, and oat groats than among chicken fed corn-based rations. It was hypothesized that the increased susceptibility of birds fed diets based on wheat may be due to indigestible cell-wall components, primarily complex carbohydrates that are present in wheat but not in corn. Diets based on rye depress chick growth and increase the number of bacteria adhering to the lower part of the intestine. It was suggested that this might be related to the relatively high content of complex carbohydrates in rye. Wheat, rye, barley, and oat groats contain variable levels of complex carbohydrates, including arabinoxylans and ß-glucans, which may interfere with digestion. Addition of enzymes such as pentosanase to rations containing these grains has improved digestibility but did not reduce the susceptibility to necrotic enteritis. The alternative hypothesis that carbohydrates in corn may be less available to microbial digestion than those in wheat is supported by the increased mortality in the corn-based ration to which glucose had been added; however the mortality was very low.

2.4.2 Influence of dietary proteins on the gut flora of chicken

TAKAHASHI et al. (1983) studied the influence of diets low in protein or lysine on the intestinal flora of chicks with reference to crop and caecal contents (table 4, 5 and 6).

Table 4. Microbial flora of crop of chicks fed on a low and normal protein diet

Bacterial group	Low protein	Normal protein
Total bacteria	8.59±0.46[a]	8.61±0.74
Enterobacteriaceae	7.94±0.93	7.64±0.11
Streptococcus	6.57±0.52	6.97±1.37
Enterococcus	3.83±0.58	4.73±1.04
Lactobacillus	8.30±0.49	8.16±1.20
Bifidobacterium	7.18±1.67	7.67±0.06
Eubacterium	6.66±0.98	5.71±3.65
Bacteroidaceae	5.31±0.46[a]	7.45±0.44
Peptococaceae	6.19±0.04	7.80±0.42
Clostridium	5.32±2.28	7.25±0.60

a Mean ± s of log bacterial count/g (when present).

Table 5. Microbial flora of caecum of chicks fed on a low or normal protein diet

Bacterial group	Low protein	Normal protein
Total bacteria	11.28±0.20[a]	11.31±0.33
Enterobacteriaceae	10.08±0.65	9.66±0.82
Streptococcus	8.61±0.56	8.89±0.90
Enterococcus	3.69±0.36	3.73±0.57
Lactobacillus	8.41±1.33	7.88±1.59
Bifidobacterium	10.44±0.24	10.29±0.83
Eubacterium	10.40±0.52	10.39±0.93
Bacteroidaceae	10.84±0.33	10.90±0.40
Peptococaceae	10.18±0.63	10.00±1.25
Clostridium	8.92±1.20	9.03±0.71
Gemmiger	9.77±0.27	9.96±0.21
Curved rods	9.52±0.27	9.54±0.53

a Mean ± s of log bacterial count/g (when present).

Table 6. Microbial flora of caecum of chicks fed on a low or normal lysine diet

Bacterial group	Low lysine	Normal lysine
Total bacteria	10.56±0.34	11.31±0.33
Enterobacteriaceae	8.34±0.21	10.42±0.84
Streptococcus	8.16±1.15	9.28±1.69
Yeast	-	6.45±3.75
Lactobacillus	3.5	5.95±2.16
Bifidobacterium	9.20±0.20	10.20±0.15
Eubacterium	9.62±0.61	10.60±0.42
Bacteroidaceae	10.10±0.27	11.00±0.35
Peptococaceae	9.72±0.37	9.88±0.40
Clostridium	4.70±2.29	6.10±2.42
Gemmiger	9.55±0.15	10.70±0.72
Curved rods	9.05±0.25	10.10±0.15

a Mean ± s of log bacterial count/g (when present).

The caecal flora of chicks fed on the low protein or low lysine diet was similar to that of chicks fed on a normal diet, but the total count of bacteria, *Eubacterium* and *Enterobacteriaceae* in the caecal content of chicks fed on the low lysine diet containing a formulated amino acid mixture minus lysine was significantly lower than that of chicks fed on the control diet. The counts for the other bacterial groups (*Bifidobacterium, Peptoccocaceae, Clostridium, Gemmiger* and curved rods) under the condition of the low lysine diet seemed to be lower than those on feeding the normal diet. The total count of *Lactobacillus* in the caecum was remarkably reduced by feeding the amino acid diet. Levels of most free amino acids in the caecal contents of the low protein group were significantly lower than those of the control. Lysine, Leucine, phenylalanine, methionine, histidine, glycine and tyrosine of the caecal contents in the low lysine group were significantly lower than those of the normal group (TAKAHASHI et al. 1983).

2.4.3 Effect of fats

Both the amount and type of dietary fat modulate intestinal immune function. Absorption of long-chain fatty acids stimulates lymphocyte flux and lymphocyte blastogenesis in intestinal lymphatics. Long-chain fatty acid absorption also significantly enhances migration of T lymphocytes to Peyer's patches, possibly due to up-regulation of adhesion molecules, such as alpha4-integrin and L-selectin. Lipoproteins are involved in stimulation of lymphocyte function by both receptor-dependent and independent mechanisms. However, unsaturated fatty acids at higher concentrations have a suppressive effect on cell-mediated immunity via eicosanoid release, receptor affinity changes or interactions with intracellular signal transduction. Fat absorption also influences various other cells in the intestinal mucosa: increased cytokine release from intestinal epithelial cells

follows long-chain fatty acid absorption. Dietary oleic acid supplements caused an immunological reversal effect in the intestinal immune system of animals fed an elemental diet. Because dietary fat intake is closely associated with immunological function of the intestinal mucosa, careful manipulation of dietary fat can be important in management of this disease (MIURA et al. 1998).

The effects of dietary fat and dietary fiber (DF) levels in diet on faecal flora, activities of three faecal enzymes, putrefactive metabolites, faecal mutagenicity and faecal properties were studied in eight healthy volunteers. They were given low fat and low DF diet (LF: fat energy ratio was 13.9%, and DF intake was 9.0 g/day) for 10 days, high fat and low DF diet (HF: fat energy ratio was 52.7%, and DF intake was 7.1 g/day) for 10 days, and high fat and high DF diet (HF: fat energy ratio was 52.0%, and DF intake was 24.8 g/day) for 10 days. No change of faecal flora at the bacterial group level was observed throughout the experimental period, except that the population of lactobacilli showed a tendency to increase in HF period. Faecal activities of beta-glucuronidase, beta-glucosidase and nitroreductase and some putrefactive products were unchanged between LF and HF, while these values decreased in HF period. No significant change of faecal properties was observed between LF and HF, while by HF supplementation faecal weight increased and faecal pH value was lower than that in LF and HF. Excretions of iron, zinc and calcium in faeces did not increase by high DF supplementation (SUGAWARA et al. 1992).

ALLEN et al. (1998) investigated the anticoccidial activities of various natural products that have potential use as dietary supplements for coccidiosis control. Sources of fats containing high concentrations of linolenic fatty acid such as menhaden oil and flaxseed (linseed) oil and flaxseed, when added to starter rations and fed to chicks from one day of age effectively reduce lesions caused by the caecal parasite *Eimeria tenella*, but not lesions caused by *Eimeria maxima*. These results are consistent with reports of effects of diets high in linolenic fatty acid on other protozoan parasites, which suggest that the state of oxidative stress induced by these diets in the cells of both host and parasites is responsible for their parasitic actions. The mechanism of its action is also considered to involve induction of oxidative stress. Diets supplemented with 8 ppm gamma-tocopherol (abundant in flaxseeds) or with 1% of the spice tumeric, reduce mid-small intestinal lesion scores and improve weight gains during *E. maxima* infections.
Flaxseed (Linseed) considered as Functional Food for people and other animals. It contains large amounts of OMEGA 3 fatty acid, alpha linolenic acid an essential fatty acid (EFA), that animal bodies can't make from other foods and very high amounts of soluble and insoluble dietary fiber and other nutrients such as proteins, carbohydrates, minerals. Flaxseed is very high in potassium. EFAs are not made by a birds' body and must be supplied daily through food supplement. EFAs are a very important part of a stress reduction program. Flaxseed favourably influences immune response. The flaxseed component, alpha linolenic acid, alters membrane phospholipids, inhibits arachidonic acid biothsynthesis from linoleic acid, and inhibits the production of proinflammmtory eicosanoids from arachidionic acid (LEAF and WEBER 1988).

Volatile and non-volatile short chain fatty acids are present throughout the intestinal tract as by-products of the metabolism of anaerobic bacteria and have been shown by several investigators to be inhibitory to a wide variety of bacteria. For example, in the presence of adequate concentrations of nutrients, the inhibition of *S.* flexneri by coliforms in broth cultures is due to volatile fatty acids production (primarily formic and acetic acids) and a concomitant decrease in the oxidation-reduction potential of the medium. The caecal contents of healthy animal contain considerable quantities of volatile fatty acid at an Eh of ca. –0.2 V. A similar environment in vitro inhibits the growth of *S.* Typhimurium and is weakly bactericidal. When the normal bacterial flora, consisting largely of obligate anaerobes, is eliminated by giving streptomycin, the Eh rises to ca. +0.2 V. and the concentration of volatile fatty acid falls, so producing conditions favo1urable to the growth of salmonellae (MEYNELL 1963). Either factor alone has some antagonistic effect, but the two factors function synergistically to produce a bactericidal effect against *Shigella* spp.

BOHNHOFF et al. (1964) showed that a similar mechanism exists for the inhibition of *S.* Enteritidis in the intestinal tract of mice. The quantity of volatile fatty acids present in the normal mouse caecum is sufficient to prevent the multiplication of *S.* Enteritidis in vitro and the inhibitory activity was greatest at low pH and Eh levels. The pH of the environment was extremely important in all these experiment since at pH levels above 7.0, the volatile fatty acids are primarily in the dissociated state and unable to inhibit the growth of enteric pathogens.

The fact that it is much easier to infect a chick during the first few days of life may be due to the low concentrations of volatile fatty acids (VFA's) present which are insufficient to prevent *Salmonella* multiplications. The high pH during the first few days after hatching renders VFA's even less inhibitory. The concentrations of the VFA's will vary considerably according to the numbers and types of anaerobes. Organisms, including faecal enterococci, which were capable of utilizing uric acid, were present in high numbers immediately after hatching (MEAD and ADAMS 1975). For the first 21 days after hatching there was a steady increase in the concentration of acetic, propionic and butyric acids in the caeca, which corresponds with the gradual establishment of the caecal flora (BARNES et al. 1979).

2.4.4 Effect of medicated ration and growth promoter factors

Antibiotic are now widely used as feed additives to promote growth or to increase feed efficiency besides prophylactic and therapeutic purpose in livestock production. There are two major effects of an antibiotic: therapeutically, it treats the invading infectious organism, but it also eliminates other, or non-disease producing, bacteria in its wake. The later do, in fact, contribute to the diversity of the ecosystem and the natural balance between susceptible and resistant strains. The consequence of antibiotic use is, therefore, the disruption of the natural microbial ecology. The alteration may be revealed in the emergence of types of bacteria which are very different from those previously found there, or drug resistant variants of the same ones that were already present. The dominance acquired by these new strains in the treated environment is directly linked to the intrinsic or acquired

resistance to the antibiotics being used. A prospective study was undertaken to determine whether feeding farm animals' antibiotics in feed caused changes in the intestinal bacterial flora of farm dwellers and their neighbours. Chickens were fed tetracycline-supplemented feed (tet-feed), and, as expected, within one week their intestinal flora contained almost entirely tetracycline-resistant organisms. Increased numbers of resistant intestinal bacteria also appeared, but more slowly, in farm members, but not their neighbours. Within five and six months, 31.3 per cent of weekly faecal samples from farm dwellers contained greater than 80 per cent tetracycline-resistant bacteria as compared to 6.8 per cent of the samples from the neighbours. Seven of the 11 farm members, but only three of the 24 neighbours, had two or more faecal samples containing greater than 80 per cent tetracycline-resistant coliforms. These resistant bacteria contained transferable plasmids conferring multiple antibiotic resistances. Selective pressure by tet-feed for antibiotic-resistant bacteria in chicken extends to human beings in contact with chicken and the feed (LEVY et al. 1976). The emergence of resistant bacteria raises concern about the bacteria and their progeny and also the extent that they spread to other environments. They have extrachromosomal replicating genes called plasmids and their associated transposons, which allow rapid and very broad dissemination genes. Gene transfer crosses species and genous barriers (DEFLAUN and LEVY 1989). Thus, resistant enterococci selected in one environment can pass resistance genes not only to other genera. Enterococci share their plasmids with *Listeria*; *E. coli* can share genes with other members of the *Enterobacteriacaece* as well as the pseudomonads and *Neisseria,* just to mention a few. In fact, the same tetracycline resistance determinants can be found among gram-positive and gram-negative bacteria as well as in the mycobacterium (ROBERTS 1997). Studies carried among chickens excreting *E. coli* with multi-resistance plasmids proved that, they did not lose the *E. coli*, despite multiple cleanings of the cage over several months. When the cages were relocated to different sites around the barn, the surrounding environment was altered and the chicken flora slowly returned to a more susceptible ones. In another study, four chickens excreting a resistant flora were added to 10 other chicken excreting a susceptible flora. Resistance was lost, the susceptible flora won out (LEVY 1985). One way is to remove or adjust the selection process so as to allow the susceptible strains to regain their former dominance. Such reversals are possible and provide the necessary optimism. There still are sufficient susceptible bacteria in our environment which, when a given chance, can return and reestablish the susceptible flora and to restore the original microbial balance between the susceptible and resistant which has been devastatingly altered by the inappropriate and continued application of antibiotics to our environment. The potential public health consequences of this use have been debated. Accumulating evidence now indicates that the use of glycopeptides avoparcin as a growth promoter has created in a food animals a major of *E. faecium*, which contains high level glycopeptides resistance determinant vanA, located on the Tn1546 transposon. Furthermore, glycopeptides-resistant strains, as well as resistance determinant, can be transmitted from animals to human *E. faecium* has the ability to cause a wide range of infections, primarily serious infections in hospital patients (particularly in intensive care units). Enterococci are resistant to many antibiotics. In Germany was shown that resistant strain of *E. faecium* could be cultured frequently from pigs, poultry, and humans suggested that resistant strain of *E. faecium* associated

with the use of glycopeptides as growth promoters in food animals. The antibiotic as selective agent and the resistance gene as the vehicle of resistance are the two most important. Both are needed in order for a clinical problem to arise, given sufficient time and quantity of antibiotic, drug resistance will eventually appear. The resistance problem is ecological, in the framework of natural competition between susceptible and resistant bacteria, antibiotic use has encouraged growth of the resistant strains, leading to an imbalance in prior relationships between susceptible and resistant bacteria. To restore efficacy to earlier antibiotics and to maintain the success of new antibiotics that are introduced, we need to use antibiotics in a way, which assures an ecological balance that favours the predominance of susceptible bacterial flora. In one study, eight poultry flocks raised conventionally and six raised without growth promoters were compared (AARESTRUP 1995). No resistant *E. faecium* was found in birds raised without growth promoters, whereas five out of eight conventional flocks contained resistant *E. faecium*. Isolation rates in positive flocks were as follows: of five faecal samples tested, one to four (20%-80%) was positive. In broilers farm where avoparcin was used, vancomycin resistant *E. faecium* was isolated from 11 of 12 faecal samples. In farms where avoparcin was not used, vancomycin resistant *E. faecium* was isolated from 2 of 12 samples. They concluded that antimicrobial agents should not be used for growth promotion if they are used in human therapeutics.

Many hypotheses have been proposed on the mechanism of growth promotion by using antibiotics as feed additives. The hypotheses proposed on the role of feed additives antibiotics by VISEK in 1978 are as follows:

1. Microorganisms responsible for infections too few to be recognized may be suppressed.
2. The microbial production of growth-depressing toxin may be reduced.
3. Antimicrobial agents may reduce the microbial destruction of essential nutrients in the gastrointestinal tract, or there may be an increase in synthesis of vitamins or other growth factors.
4. The efficiency of absorption of nutrients may be enhanced, because the intestinal wall is rather thin.

Many workers evaluated the significance of the microflora in poultry during the administration of dietary antibiotics. Most of them, however, committed two common defects. One defect is that the methods of culture employed for anaerobic microorganisms which are predominant in the intestinal microflora, were inadequate. The other is that the experimental period used was comparatively short. An attempt was made by OHYA and SATO (1983) to reveal changes in the intestinal flora in broiler chicken fed a diet containing antibiotics for 6 weeks. At the same time, three antibiotics, colistin (CL) in 5, 50 and 100 mg/kg ration, bacitracin (BC) in 4.8, 48 and 96 mg/kg and enramycin (ER) in 2, 20 and 40 mg/kg were used. They are not absorbed from the intestinal wall and widely used as feed additives. Significant changes in microflora were observed mainly in such bacterial groups as aerobic bacteria and *Lactobacillus.* Food experiments were carried out:

In the CL-5 and CL-50 groups, significant changes in the microflora were seen in bacterial groups including *Enterobacteriaceae, Enterococcus, and Lactobacillus*, total aerobes, total anaerobes, and total bacteria. In the small intestine *Enterobacteriaceae* increased in CL-5, at 4 weeks of age, but decreased in CL-50 at 6 weeks of age. *Lactobacillus* increased in CL-5 at 1 week of age, but decreased in CL-5 at 3 weeks of age. *Enterococcus* decreased in CL-5 at 6 weeks of age. Total aerobes increased in Cl-5 at 2 weeks of age. On the other hand, total anaerobes increased in CL-5 at 1 week of age and decreased at 3 weeks of age. In the caecum *Enterobacteriaceae* increased in Cl-5 at 2 weeks of age. An increase in *Enterococcus* was obvious in CL-5 and CL-50 at 1 week of age. *Lactobacillus* increased in CL-5 and CL-50 at 1 week of age, but decreased in CL-5 at 3 week of age. No changes were observed in the number of total aerobes, total anaerobes, or total bacteria.

Changes in the microflora of the BC-4.8 and BC-48 were observed mainly in *Enterobacteriaceae, Enterococcus*, *Lactobacillus*, total aerobes, total anaerobes and total bacteria. In small intestine *Enterococcus* and *Lactobacillus* decreased in BC-48 at 1 week of age. As the reflection of these changes, total aerobes, total anaerobes and total bacteria decreased in this group. A similar variation was observed at 3 weeks of age. *Enterobacteriaceae* increased in BC-48 at 2 weeks of age. *Lactobacillus* increased in BC-4.8 at 2 weeks of age. A decrease in *Enterococcus* was obvious in BC-48 at 4 weeks of age. In the caecum *Enterobacteriaceae* increased in BC-48 throughout the experimental period. *Lactobacillus* increased in BC-4.8 and BC-48 at 2 weeks of age. An increase in total aerobes was observed in BC-48 at 2 and 3 weeks of age.

Essentially variation in bacterial groups was seen in ER-2 and ER-20 during the experimental period as in the groups CL and BC. In the small intestine *Enterococcus*, total aerobes and total bacteria increased in ER-20 at 1 week of age. *Lactobacillus*, total aerobes and total bacteria decreased in ER-20 at 3 week of age. An increase was observed in total anaerobes and total bacteria in ER-2 at 4 weeks of age. *Lactobacillus* decreased in ER-20 at 3 weeks of age. The occurrence of recovery of *Micrococcaceae* decreased in ER-2 and ER-20 at 3 weeks of age. In the caecum *Enterobacteriaceace* increased ER-2 and Er-20 at 1 week of age, and in ER-20 at 2 week of age. An increase in *Enterococcus* was observed in ER-20 at 1 week of age and in ER-2 at 3 weeks of age. *Micrococcaceae* decreased in ER-2 at 4 weeks of age. Changes in microflora induced by the antibiotics were focused on such bacterial groups such as *Enterobacteriaceace*, *Enterococcus*, *Lactobacillus*, total aerobes, total anaerobes and total bacteria.

Changes in intestinal microflora in short-term administration one day after administration (13 days of age): In the small intestine *Enterobacteriaceace*, *Enterococcus* and *Lactobacillus* decreased in the CL, BC and Er groups respectively. In the caecum *Enterococcus*, increased in the CL groups, and *Enterobacteriaceace* in the BC groups. A decrease in *Lactobacillus* was noticed in the ER diet seven days after administration (19 days of age): In the small intestine *Enterococcus*, *Lactobacillus* and *Entrobacteriaceace* decreased in CL, BC and Er groups respectively. In the caecum *Enterococcus* decreased and *Clostridium* increased in the CL groups. A decrease in

Bacteroidaceace and an increase in *Clostridium* were obvious in the ER group. The effect of the antibiotic on the intestinal microflora was seen even 7 days after administration.

In the long-term administration with the antibiotics, the intestinal microflora showed significant changes mainly over a period from 1 to 3 weeks of age. It is well known that a microflora is established 2 to 3 weeks after hatching (BARNES 1972 and OCHI et al. 1964). Antibiotics contained in feed may affect the establishment of intestinal microflora in broiler chicken. The variance of intestinal microflora induced by the dietary antibiotic is assumed to be composed of the following three phenomena: -

1. Changes directly related to antibacterial spectrum of the antibiotic concerned.
2. Antagonistic changes related to an ecological balance in the bacterial flora.
3. Changes in the quantitative balance of bacteria, which constitute each bacterial group.

By this regards it will be concluded that the changes in intestinal microflora during the administration of an antibiotic diet will be expressed as complicated form of the three possible modes of transition of the microflora. It was reported that the use of antibiotic such as oxytetracycline could cause an inhibition of the immune system (FORSGREN and GNARPE 1978). Furthermore, the feeding of chlorotetracycline resulted in increased shedding of salmonellae and enhanced the severity of infection (DEY et al. 1978). The effect of Terramycin, administered prophylactically in drinking water, on the gut flora of broiler birds was investigated. Exposure to the antibiotic for only 24 h profoundly affected the counts of tetracycline-resistant strains and selected O-serotypes carrying resistance determinants. Large numbers of *E. coli* resistant to sulphonamides were found in treated and control birds and this is discussed in relation to the use of sulphaquinoxaline as a coccidiostat. Evidence of carcass contamination by antibiotic resistant *E. coli* found in the gut is presented (HOWE and LINTON 1976). Administration of 0.05 g/kg oxytetracycline or 0.05 g/kg sulphadimidine in feed to broiler chicks for 50 days caused a significant decrease of the total number of leukocytes, lymphocytes and the size of BF and thymus but not spleen or body weight. The antibacterial significantly reduced the macrophage phagocytic activity. It suggested that the prolonged administration of oxytetracycline and sulphonamide to chicken might induce an immunosuppressant effect (AL-ANKARI and HOMEIDA 1996).

2.4.5 Influence of dietary probiotic on the gut flora of poultry

2.4.5.1 The concept of bacterial interference and competitive exclusion

The mechanisms used by one species of bacteria to exclude or reduce the growth of another species are varied, but ROLFE (1991) determined that there are at least four major mechanisms

1) Creation of microecology that is hostile to another bacterial species.

2) Elimination of available bacterial receptor sites.

3) Production and secretion of antimicrobial metabolites.

4) Selective and competitive depletion of essential nutrients.

The biological activity of probiotic bacteria is due in part to their ability to attach to enterocytes. This inhibits the binding of enteric pathogens by a process of CE. Attachment of probiotic bacteria to cell surface receptors of enterocytes also initiates signalling events that result in the synthesis of cytokines. Probiotic bacteria also exert an influence on commensal micro-organisms by the production of lactic acid and bacteriocins. These substances inhibit growth of pathogens and also alter the ecological balance of enteric commensals. Production of butyric acid by some probiotic bacteria affects the turnover of enterocytes and neutralizes the activity of dietary carcinogens, such as nitrosamines, that are generated by the metabolic activity of commensal bacteria in subjects consuming a high-protein diet (KAILASAPATHY and CHIN 2000).

A probiotic is a viable microbial dietary supplement that beneficially affects the host through its effects in the intestinal tract. Several health-related effects associated with the intake of probiotics, including alleviation of lactose intolerance and immune enhancement, have been reported. RANTALA and NURMI (1973) found that pretreating chicks with flora isolated from the alimentary tract of adult chicken reduced the colonization of *Salmonella* Infantis in the caeca (RANTALA and NURMI 1973). Further work conducted by BARNES et al. (1979) indicated that avian gut isolates exerted anti- *Salmonella* activity in chicks.

SNOEYENBOS et al. (1978, 1985) recorded that resistance of young chicks and poults to *Salmonella* exposure was substantially increased by early oral administration of intestinal contents or faeces from selected adult chicken. Protection was secured also by administering anaerobic broth cultures of intestinal microflora from selected donor birds. Protection was substantial for 63 days, the longest period tested, although it could be overcome by severe exposure. The protective mechanism appears to be a consequence of CE of *Salmonella* by "normal" microflora of the gastrointestinal tract. CE of *Salmonella* by native gut microflora was studied in 24 groups of 100 chickens each started in thoroughly cleaned and sanitized isolation facilities. During 53-days test periods, infection by both *Salmonella* Infantis and *S.* Typhimurium was greatly restricted in groups previously treated with native microflora compared with control groups. Feed and water starvation for 48 hours starting at either 23 or 51 days did not affect the incidence of infection in protected groups. The protective flora spread readily to adjacent untreated groups, infected groups given the protective flora at 11 days exhibited a more rapid elimination of infection than untreated control groups.

S. Typhimurium colonizes the intestinal tract of poultry and causes food-borne illness in humans. Reduction of *S.* Typhimurium colonization in the intestinal tract of poultry reduces potential carcass contamination during slaughter. Limited evidence on the nature of CE organisms has indicated that *Enterococcus*, *Veillonella, Bacteriodes, Bifidobacterium*, *Enterobacter, Fusobacterium, Escherichia, Lactobacillus, Eubacterium,* and *Propionibacterium* species may be effectors of CE. However, most reports have indicated that complex mixtures of these bacteria plus other undefined organisms are required for effective CE. Fresh caecal material is usually more effective than frozen or lyophilized material, as cultures derived from mature chicken's caecal contents lose the ability to affect colonization control during storage (SNOEYENBOS et al. 1985).

PIVNICK and NURMI (1982) found that one CE culture stored at –70 C was as protective as fresh CE, but another culture that had been stored for 5 months at –70 C was not as effective as fresh CE. When chicks treated with ceacal microflora, derived from sequential passage through either broilers or layers, were subsequently challenged with oral inoculation of non lactose-fermenting *S.* Typhimurium, the treatments caused a significant reduction in caecal concentration of *Salmonella*. Best results were obtained when the chicks were fed a diet containing lactose 5% as the combination treatment with lactose and probiotic resulted in a markedly reduced caecal pH and a sharp increase in the caecal concentration of undissociated propionic acid, a substance known to be bactericidal (ZIPRIN and DELOACH 1993).

PROMSOPONE et al. (1998) studied the effect of an avian-specific probiotic and *S.* Typhimurium-specific antibodies on the colonization of *S.* Typhimurium in broilers and on body weights. Broiler chicks were spray-vaccinated at the hatchery with the commercial product. Avian Pac Plus, which contains *L. acidophilus, E. faecium*, *S.* Typhimurium-specific antibodies. At placement, these chicks were administered Avian Pac Plus in the water. Six hours postplacement, chicks were orally challenged with 1.8×10^{7} CFU of *S.* Typhimurium. Chicks were administered Avian Pac Plus for two additional days postchallenge. Chicks were evaluated for *S.* Typhimurium colonization and shedding every 3 to 4 days for the first 2 weeks and every 7 days for 6 weeks. The mean caecal and colonic concentration of *S.* Typhimurium from the Avian Pac Plus-treated group was significantly lower at day 31, day 38, and day 43 than the nontreated control group. These results indicated that a combination of *L. acidophilus*, *E. faecium*, and *S.* Typhimurium-specific antibodies have a beneficial effect in reducing the colonization of *S.* Typhimurium in market-aged broilers.

There are many reports suggesting that lactobacilli are important in creating a balanced microbial population in the intestinal tract. The chicken is a unique experimental animal to examine this phenomenon. Its crop characteristically contains a microflora in which the lactobacilli predominate over coliforms and Enterococci. This population of lactobacilli is in a position to influence not only the microflora of the crop but also that of small intestine. Epithelial adhesion enables the lactobacilli to remain in large numbers after the food has left the crop and persist even after period of fasting. Thus there is always a large population of lactobacilli to inoculate the incoming food to ensure that the lactobacilli are not only dominant but present in sufficient numbers to suppress multiplication of *E. coli*. The production of pH due to lactic acid is sufficient to suppress the growth of *E. coli* and many other organisms, including lactobacilli themselves, but not yeasts. Compared with the neonatal pig and calf, the chickens are resistant to *E. coli* enteric infections. It is temping to relate this to the presence of inhibitory *Lactobacillus* flora in the anterior part of chicken gut. Thus the delayed colonization of the crop by *Lactobacillus*, which can occur, for example in a clean environment, may render the chickens more susceptible to clinical and subclinical infections. Moreover, healthy chicks given *Lactobacillus* per os soon after hatching showed an improved growth rate in the first 3 weeks of life. This may be indication that even under conditions of more severe bacteriological stress, such as disease, chicks would perform better if lactobacilli were established in the crop immediately after hatching (FULLER 1977).

LAROUSSE (1970) indicated that *L. acidophilus* inoculation resulted in equal or superior growth performance in chicks given antibiotics as growth promotants. Also TORTUERO (1973) determined that *L. acidophilus* inoculation to day-old broiler chicks improved weight gain, feed conversion, fat digestibility, and nitrogen retention. Additionally, caecal weight was reduced and the production of caecal material (the prime source of carcass microbiological contaminants) was reduced significantly. Along with these determinations was the observation that intestinal microflora was altered significantly in that there was a significant increase in *Lactobacillus* colonies and a total decrease in enterococci colonies.

Experiments were conducted to evaluate the use of lactobacilli in poultry medicine. These were the findings: the numbers of lactobacilli and *E. coli* present in the caeca of newly hatched chicks from the same hatchery had marked variation among hatches, lactobacilli inoculated into the crop a few hours after hatching did not induce a reduction of *S.* Infantis in the caecal contents, a single dose of lactobacilli given at one day after hatching did not appear to alter the lactobacilli -coliform balance in the cecum, in one group of fasted chicks, lactobacilli inoculation prevented accumulation of excreta around the vents, prophylactic feeding of the bacillus to non-stressed chicks averted pasted vents, continuous feeding of lactobacilli produced a numerical increase of body weight that was not statistically significant in the small groups of birds utilized (ADLER and DA MASSA 1980).

FRANCIS et al. (1978) observed increased growth in turkeys fed *L. acidophilus* and other *Lactobacillus* cultures and zinc bacitracin. Both *Lactobacillus* and zinc bacitracin decreased the coliform and total aerobic counts in the feed and in the intestinal tract of the poults, and zinc bacitracin also decreased intestinal *Lactobacillus* colonies. SOERJADI et al. (1981b) investigated the CE of salmonellae by native gut microflora by treating chicks with various avian lactobacilli. The evaluation of protection was based on the number of salmonellae adhering to the mucosa of the crop and the caecum, enumeration of salmonellae in faecal droppings, and enrichment of cloacal swabs and faecal droppings using both individual and seeder bird tests. Lactobacilli reduced the number of salmonellae adhering to the crop mucosa by 1 to 2 logs. Treatment with lactobacilli did not lower the number of chicken shedding salmonellae or reduces the number of salmonellae adhering to the mucosa of the cecum. Lactobacilli as a single bacterial treatment played a minor role in protecting the crop, but no protection of the caecum was demonstrated.

Chicks were hatched germ-free in gnotobiotic isolators to determine the inhibitory effects of *L. acidophilus* towards pathogeneic *E. coli* in vivo. Twelve trials were conducted in two flexible film isolators utilizing a total of 221 chicks. One treatment consisted of inoculating 2-day-old chicks with *L. acidophilus*, then challenging with pathogenic *E. coli* with subsequent dosing with L. acidophilus. The other treatment consisted of challenging with the *E. coli* at 2 days of age, then subsequently dosing with *L. acidophilus*. Statistical analysis of the data showed initial dosing with *L. acidophilus* prevented excessive mortality when chicks were challenged with *E. coli*. Also, continued dosing with *L. acidophilus* lowered the pH in the crop, caecum, and rectum whether

chicks were initially given *L. acidophilus* or *E. coli*. This strain of L. acidophilus was capable of competing with *E. coli* in the gut of gnotobiotic chicks (WATKINS et al. 1982).

WATKINS and MILLER (1983) found that the shedding of pathogenic *Salmonella* Typhimurium and *S. aureus* in the faeces of gnotobiotic chicks was greatly reduced by consecutive treatment with *L. acidophilus* for both prophylactic and therapeutic treatment. A significant increase in lactobacilli shedding was observed with the decreased shedding of both pathogens. The *Lactobacillus* treatments reduced the average mortality from *Salmonella* Typhimurium from 36.7 to 8.8%. Mortality from *S. aureus* was reduced from 32.6 to 11.1% by consecutive treatment with *L. acidophilus*. CE of *S.* Typhimurium by isolates of lactobacilli was studied also in day-old chicks. Protection was evaluated by enumerating *Salmonella* in faeces and cloacal swab cultures from test chickens. Neither single nor multiple treatments with six morphologically distinct isolates of lactobacilli resulted in major protection against infection by *S.* Typhimurium (WEINACK et al. 1985).
When monoflora chickens with *L. acidophilus* or *E. faecalis* were inoculated with *C. perfringens* in broth culture, few or no chicken died. Approximately 50% of germ-free chicken died after inoculation of *C. perfringens* culture, whereas no conventional birds died after inoculation of broth culture. *C. perfringens* in the contents of duodenum from germ-free chicken numbered about 10^4 CFU/g after inoculation of 10^8 CFU in broth culture per bird, but in gnotobiotic and conventional chicken these organisms decreased or were not detected. When *C. perfringens* was cultured in intestinal contents collected from germ-free chicken, *C. perfringens* proliferated but alpha toxin was not detected. These findings indicate that the pathogenicity of *C. perfringens* was suppressed by *L. acidophilus* or *E. faecalis* administered previously or inhibited by normal intestinal flora. It is likely that when changes in intestinal enviroment take place, such as reduction of major intestinal microflora, the number of *C. perfringens* may increase, which in turn results in a greater production of alpha-toxin by *C. perfringens*, and outbreak of necrotic enteritis may be induced (FUKATA et al. 1991).

2.4.6 Influence of dietary prebiotic on the gut flora of animals

There is increasing interest in using dietary inputs to manage the GIT microecology. However the influence of diet is complex, they involve several possible mechanisms of action, all of which are not yet well understood. Dietary inputs can serve as sustrates for the bacteria. Some components of the diet can alter the assemblages of bacteria, just as adding fermentable fibers, such as oligofructose and inulin, selectively increase the abundance of lactic acid bacteria while decreasing the percentages of potential pathogens and putrefactive bacteria. They also influence the metabolic activities of bacteria. Whereas antibiotic disturb the GIT bacterial assemblages and this can affect the structure and functions of the mucosa. The changes in the microenviroment can lead to the proliferation of some pathogens, such as clostridia (BUDDINGTON and WEIHER 1999).

2.4.6.1 Prebiotic agents

Prebiotic agents are nondigestible food ingredients that are beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of gut bacteria that can improve the host health. Criteria that allow the classification of a food ingredient as a prebiotic include the following: it must be neither hydrolysed, nor absorbed in the upper part of gastrointestinal tract. It must be selectively fermented by one or a limited number of potentially beneficial gut bacteria. It must be alter the composition of gut bacteria towards a healthier composition. It must preferably induce effects that are beneficial to the host health (GIBSON 1999). Although prebiotics offer one rational approach to the probiotic concept, the health consequences have not yet been defined. In theory, a number of potential benefits may arise. However, it may be that improved resistance to pathogens offers the most feasibility. The lactic acid microflora of the gastrointestinal tract is thought to play a significant role in improved colonization resistance. Increased bifidobacterial numbers may be one factor that contributes towards improved CE of pathogens (GIBSON 1999).

The main commercial prebiotic agents consist of oligosaccharides and dietary fibres (mainly inulin). They are essentially obtained by one of three processes: the direct extraction of natural polysaccharides from plants. Inulin and oligofructose are present as plant storage carbohydrates in a number of vegetables and plants including wheat, onion, bananas, garlic and chicory, the controlled hydrolysis of such natural polysaccharides, enzymatic synthesis, using hydrolases and/or glycosyl transferases. Both of these enzyme types catalyse transglycosylation reactions, allowing synthesis of small molecular weight synthetic oligosaccharides from mono- and disaccharides. Presently, in Europe, inulin-type fructans, characterised by the presence of fructosyl units bound to the beta-2, 1 position of sucrose, are considered as one of the carbohydrate prebiotic references. Work with prebiotics has been limited, and only studies involving the inulin-type fructans have generated sufficient data for thorough evaluation regarding their possible use as functional food ingredients. The combination of probiotics and prebiotics in a synbiotic improve the survival of the bacteria crossing the upper part of the gastrointestinal tract, thereby enhancing their effects in the large intestine. In addition, their effects might be additive or even synergistic (ROBERFROID 2000).

Prebiotics induce changes in the population and metabolic characteristics of the gastrointestinal bacteria, modulate enteric and systemic immune functions, and provide laboratory rodents with resistance to carcinogens that promote colorectal cancer (BUDDINGTON et al. 2002).

Prebiotics escape enzymatic digestion in the upper gastrointestinal tract and enter the caecum without change to their structure. None are excreted in the stools, indicating that they are fermented by colonic flora so as to give a mixture of short-chain fatty acids (acetate, propionate and butyrate), L-lactate, carbon dioxide and hydrogen. By stimulating bifidobacteria, they may have the following implications for health: inhibition of bacterial enteritis (diarrhea) by inhibiting putrefactive bacteria (*C. perfringens*) and pathogenic bacteria (*E. coli, Salmonella, Listeria* and *Shigella*), respectively,

improvement of lipid metabolism, fibre-like properties by decreasing the renal nitrogen excretion, 4) improvement in the bioavailability of essential minerals, in particular, calcium, and 5) Stimulation of the immune system, (GRIZARD and BARTHOMEUF 1999, ROBERFROID 1999). The use of oligofructose and inulin in diet caused a marked inrease in bifidobacteria, whereas *Bacteriodes*, fusobacteria and clostridia all decreased. Other bacteria tested (total aerobes, total anaerobes, lactobacilli, coliforms and gram-positive cocci) remained more or less unchanged (GIBSON 1999).

2.4.6.2 Inulin, oligofructose and intestinal function

Inulin and oligofructose are natural food ingredients which are receiving increased attention from the scientific community for their nutrition, physiological and prebiotic effects. The main physiological effects of inulin and oligofructose are primarily on gastric emptying and small intestinal transit time, resulting in an improved glucose tolerance. Second, on colonic transit time and large bowel functions due to fermentation by caeco-colonic microbial flora or bulking action. Inulin and oligofructose are fermented to a large extent by a wide variety of anaerobic bacteria that result in an increase in bacterial biomass, an increase in faecal mass, a change in intracolonic pH, and production of short chain fatty acids and various gases as metabolic end products. The short chain fatty acids resulting from the colonic fermentation of inulin and oligofructose are largely absorbed via the portal blood and reach both the liver and the peripheral tissues. They induce changes in glucose and fat metabolism leading to post-prandial hypoglycemia and long-term hypolipidemia. When inulin and oligofructose were added to a controlled diet, significant increases were noted in the bifidobacterial populations however, there does not appear to be an increase in total bacterial numbers or a change in anaerobe: aerobe ratio, increased vitamin B absorption resulted from increased small intestinal absorption or bacterial synthesis, increase immune function possibly due to increase serum glutamine levels which act as substrate for lymphatic tissue. It has been proposed that these changes promote both colonic and systemic health through modification of the intestinal microflora. Inulin and oligofructose are rapidly and completely fermented by the colonic microflora with the production of acetate and other short-chain fatty acids. As with lactulose, they may also result in the growth of the faecal biomass, and in doing so, entrap ammonia for bacterial protein synthesis or conversion to the ammonium ion (JENKINS et al. 1999). Inulin is a fructooligosaccharide (FOS) derived from chicory by a process similar to that used in extracting sugar from sugar beets. It is white in color, slightly hygroscopic and is water soluble and not digested by monogastric animals. It is not only a natural ingredient but also a prebiotic, which stimulates the growth of good intestinal bacteria which support healthy colon conditions. This relationship has a positive effect on the digestive system function, as well as the overall health of the animal. The inulin functions by increasing the bioavailability of minerals, particularly calcium, through its fermentation of *Lactobacillus* and bifidobacteria in the intestinal tract. Studies with rats indicated an increase in bone density after consumption of inulin/FOS. In addition, the beneficial effect of inulin and oligofructose on the presence of bifidobacteria suggests an improved absorption

of vitamins, such as the B complexes. Prebiotics induce changes in the population and metabolic characteristics of the gastrointestinal bacteria, modulate enteric and systemic immune functions, and provide laboratory rodents with resistance to carcinogens that promote colorectal cancer. There is less known about protection from other challenges. Therefore, mice of the B6C3F1 strain were fed for 6 weeks a control diet with 100 g/kg cellulose or one of two experimental diets with the cellulose replaced entirely by the nondigestible oligosaccharides (NDO) oligofructose and inulin. From each diet, 25 mice were challenged by a promoter of colorectal cancer (1, 2-dimethylhydrazine), B16F10 tumor cells, the enteric pathogen *C. albicans* (enterically), or were infected systemically with *Listeria (L,) monocytogenes* or *S.* Typhimurium. The incidences of aberrant crypt foci in the distal colon after exposure to dimethylhdrazine for mice fed inulin (53%) and oligofructose (54%) were lower than in control mice (76%) but the fructans did not reduce the incidence of lung tumors after injection of the B16F10 tumor cells. Mice fed the diets with fructans had 50% lower densities of *C. albicans* in the small intestine. A systemic infection with *L. monocytogenes* caused nearly 30% mortality among control mice, but none of the mice fed inulin died, with survival intermediate for mice fed oligofructose. Mortality was higher for the systemic infection *of S.* Typhimurium (>80% for control mice), but fewer of the mice fed inulin died, with mice fed oligofructose again intermediate. The mechanistic basis for the increased resistance provided by dietary NDO was not elucidated, but the findings are consistent with enhanced immune functions in response to changes in the composition and metabolic characteristics of the bacteria resident in the gastrointestinal tract (BUDDINGTON et al. 2002).

Inulin is major constituent of some of the most famous of the "old-standby" herbs, such as burdock root, dandelion root, elecampane root, chicory root, and the Chinese herb codonopsis. Botanically, inulin is a storage food in the plants of the Composite family. Inulin when injected interacts with complement system, which has resulted in rumors in herbal circles that it is immunostimulant. It is not digested or absorbed, however, (except perhaps in micro-amounts) and such effects are not observed with oral use. Inulin is recommended sometimes for diabetics, it has a mildly sweet taste, and is filling like starchy foods, but because it is not absorbed, it does not affect blood sugar levels. Despite the similarity of its name to insulin, inulin has no connection with that hormone either chemically or through physiological activity. Inulin is soluble in hot water, but only slightly soluble in cold water or alcohol, so is not present to any significant extent in tinctures. All the above herbs have traditionally been taken in decoctions, and in this form may deliver significant amounts of inulin. Recent research has shown an important physiological action for inulin (GIBSON 1995, ROBERFROID 1993).

Like some pectins and fructooligosaccharides, inulin is a preferred food for the lactobacilli in the intestine and can improve the balance of friendly bacteria in the bowel. Subjects in one trial were giving 15 grams of inulin a day for fifteen days. *Lactobacillus* and bifidobacteria increased by about 10% during that period. Gram-positive bacteria associated with disease declined. Bifidobacteria digest inulin to produce short chain fatty-acids, such as acetic, propionic, and butyric acids. The

first two may be used by the liver for energy production, while butyric acid has cancer-preventing properties within the intestine. Recent animal research also shows that inulin prevents precancerous changes in the colon. Inulin enhances the immune system and help with digestion by improving gut integrity and maintaining normal intestinal flora. It Decreases episodes of and minimize viral and bacterial diarrhea and inhibit the growth of many harmful bacteria and yeast, including those that cause food-borne illness like *E. coli*, *Enterococcus*, *Salmonella*, and *Listeria*. Inulin (a nondigestible fiber) is a prebiotic that helps promote the growth of these "good" bacteria in the colon. It serves as food for these organisms. The *Lactobacillus* and bifidobacteria actually digest the inulin for us and then increase as much as 5 to 10 times in volume. A synergistic relationship is developed where the whole is greater than the sum of the parts. The inulin comes from chicory root, which is extracted by a natural hot water distilling process. Historically, humans have eaten significantly large amounts of inulin. The highest food concentrations occur in dahlia tubers, burdock roots, chicory roots and greens, foods that are not traditionally eaten in large amounts currently. Sixteenth century Europeans consumed about 35 g of inulin daily, while 19th century Central and South Americans consumed up to 100 g daily. Because inulin is a soluble fiber, it helps maintain normal bowel function, decreases constipation, lowers cholesterol and triglycerides, and helps normalize blood sugar levels. Everyone would benefit from more fiber in his or her diet. This is particularly true for diabetics. Inulin doesn't raise blood sugar or require insulin to metabolize it. Despite its similarity in spelling to insulin, inulin has no connection with the hormone. Inulin has been called a fat substitute as well as a sugar substitute, but it's not an artificial chemical. In fact, its slightly sweet taste and smooth texture improves the eating experience of low fat and fat free foods. Research is demonstrating that the nutritional value of inulin goes beyond what is typical of most classical fibers (REDDY 1997).

The inhibitory effects of CE and 0.1% concentration of fructooligosaccharide (FOS), singly and in combination, on *Salmonella* colonization of chicks were investigated. One-day-old birds were divided into four groups: (i) control, (ii) CE, (iii) FOS, and (iv) CE plus FOS. Chicks received *S.* Enteritidis at 7 days (experiment 1) or 21 days (experiment 2). Birds in each group were killed at 1 day, 7 days, and 14 days after inoculation of *S.* Enteritidis for count of *Salmonella* in caecal contents. In experiment 1, the mean number of *S.* Enteritidis in the chicks inoculated with CE was significantly decreased compared with the other three groups at 1 day postinoculation. In experiment 2, the mean numbers of *S.* Enteritidis in the chicks of the FOS group and the FOS plus CE group were significantly decreased compared with the control group at 1 day and 7 days postinoculation. On 7- and 21-day-old chicks, few changes on number of total bacteria, *Bifidobacterium*, *Bacteroides*, *Lactobacillus*, and *E. coli* were observed in the caecal contents of treated groups compared with the control group. Low-dose feeding of FOS in the diet of chicks with a CE treatment may result in reduced susceptibility to *Salmonella* colonization but may not lead to a shift in the intestinal gut microflora on 7- and 21-day-old chicks. Although when chicken are fed FOS for long times, *Bifidobacterium* and/or *Lactobacillus* of the intestinal flora may increase (FUKATA et al. 1999).

BAILEY et al. (1991) investigated the influence of fructooligosaccharide (FOS) on the ability of *S.* Typhimurium to grow and colonize the gut of chicken. In vitro studies showed that *Salmonella* did not grow when FOS was the sole carbon source. When FOS was fed to chicks at the 0.375% level, little influence on *Salmonella* colonization was observed. At the 0.75% level, 12% fewer FOS-fed birds were colonized with *Salmonella* compared with control birds. When chicks given a partially protective CE culture were fed diets supplemented with 0.75% FOS, only 4 of 21 (19%) chicken challenged with 10^9 *Salmonella* cells on day 7 became colonized as compared with 14 of 23 (61%) chicken given CE alone. When chickens were stressed by feed and water deprivation on day 13 and challenged with 10^9 *Salmonella* on day 14, 33 of 36 (92%) chickens fed control diets were colonized compared with only 9 of 36 (25%) chickens fed a 0.75% FOS diet. Chickens treated with FOS had a fourfold reduction in the level of *Salmonella* present in the caeca. Feeding FOS in the diet of chicken may lead to a shift in the intestinal gut microflora, and under some circumstances may result in reduced susceptibility to *Salmonella* colonization.

Generally, fiber and compounds associated with fiber in cereal products (e.g., phytates) have been found to reduce the apparent absorption of minerals (such as calcium, magnesium, zinc and manganese) in humans, livestock and animal models. The addition of soluble forms of fiber (specifically pectins, gums, resistant starches, lactulose, oligofructose and inulin) has been found in various studies to promote fermentation and the production of volatile fatty acids in the cecum, have a trophic effect on the caeca of animals and improve absorption of minerals. This may reflect absorption of electrolytes from the large intestine (GREGER 1999).

Fructooligosaccharides (FOS) have been shown to be nondigestable in humans and animals (OKU et al. 1984), and have been shown to be utilized by only a few pathogenic and non-pathogenic intestinal bacteria in pure culture (HIDAKA et al. 1986). FOS are not hydrolysed by digestive enzymes, but utilized by intestinal bacteria for the production of carbon dioxide and organic acids. Therefore, growth of natural microflora able of inhibiting enteropathogen growth in the chicken gastrointestinal tract may be promoted by addition of dietary FOS. *Bifidobacterium* spp., peptostreptococci, and *Klebsiella* utilize FOS, but other bacteria particularly *C. perfringens, E. coli,* and *Salmonella* spp. do not. BAILEY et al. (1991) tested 20 serotypes of *Salmonella* for growth in minimal medium containing pure FOS as the only carbohydrate source. None of the serotypes grew in the presence of FOS. Fructooligosaccharides tend to selectively enrich for beneficial genera such as lactobacilli and bifidobacteria (HIDAKA et al. 1986). Feeding fructooligosaccharides have been shown to improve animal performance, reduce serum cholesterol, reduce disease-related diarrhea, alleviate constipation, reduce intestinal concentrations of putrefactive compounds, reduce tumors, and inhance the immune response in a number of species (HIDAKA et al. 1986). Studies reporting the use of fructooligosaccharides in poultry diets have indicated improvements in weight gain and feed efficiency, reduction in mortality, and reduction in intestinal colonization by *Salmonella* (AMMERMAN et al. 1988, 1989, BAILEY et al. 1991, FARNWORTH et al. 1993, WALDEROUP et al. 1993).

Feeding lactosucrose to broilers increased numbers of bifidobacteria, decreased concentration of putrefactive products (phenol and p-cresol) and ammonia, and increased the concentration of volatile fatty acids in broiler faeces. Thus, oligosaccharides may potentially be useful in reducing ammonia and other environmental odours emanating from poultry production facilities. Bifidobacteria are recognised to have beneficial effects on digestive disorders. They have been shown to protect gnotobiotic quails against necrotizing enterocolitis-like lesions when the birds were inoculated with faecal flora from preterm infants, decreasing the clostridial population. Experiments were done in eight groups of germ-free quails for 28 days. The groups differed as to their bacterial status, diet and environment. Quails were inoculated with one of two floras from premature twins. The first flora included *Bifidobacterium* pseudo-catenulatum, *E. coli* and no clostridia. The second flora included clostridial species and was associated with *B.* Infantis-Longum. Caecal bacterial population and metabolism changes were investigated with lactose (6%) diet versus a lactose-oligofructose (3%-3%) diet, either in a gnotobiotic environment or in an ordinary environment permitting post-colonisation by exogenous bacteria. In both environments and with both flora, oligofructose significantly increased the level of bifidobacteria and this was associated with a decrease of *E. coli* or *C. perfringens* and *C. ramosum* (TERADA et al. 1994).

The bacterial changes in the ordinary environment depended on the initial composition of the microflora and the colonization resistance against exogenous bacteria was more efficient with the flora that included *B.* pseudo-catenulatum. The changes in caecal pH and short-chain fatty acids were minimal. It was demonstrated that, irrespective of the environmental conditions, the use of oligofructose helped to prevent the overgrowth of bacteria implicated in digestive disorders (CATALA et al. 1999).

The in vitro fermentability of oligofructose and inulin was compared with a range of reference carbohydrates by measuring bacterial end-product formation in batch culture. Short chain fatty acid and gas formation indicated that these substrates, which occur naturally in the diet and reach the colon in a largely intact form, were utilized by mixed populations of gut bacteria. Bacterial growth data showed that oligofructose and inulin exerted a preferential stimulatory effect on numbers of the health-promoting genus *Bifidobacterium*, whilst maintaining populations of potential pathogens (*E. coli, Clostridium*) at relatively low levels. *Bifidobacterium* was able to exert an inhibitory effect not necessarily related to acid production. Further studies showed that eight species of bifidobacteria could variously excrete an anti-microbial substance with a broad spectrum of activity. Species belonging to the genera *Salmonella*, *Listeria, Campylobacter* and *Shigella*, as well as *Vibrio cholerae*, were all affected. Bifidobacteria are able to exert more than one mechanism of inhibition, which may be of some importance with regard to protection against gastroenteritis. Pure culture studies confirmed the enhanced ability of bifidobacteria to utilize these substrates in comparison with glucose. Batch culture experiments demonstrated that the growth of *Bifidobacterium* Infantis had an inhibitory effect towards *E. coli* and *C. perfringens*.

Potentially, an increase in the concentration of these substrates in the diet may therefore improve the composition of the large intestinal microflora and have positive effects on the quality of the diet (WANG and GIBSON 1993, 1994).

2.5 Interference of *Bdellovibrio* with intestinal flora

2.5.1 Habitat and growth conditions

Bdello is Greek for leech and the leech-like *Bdellovibrio* was the first predatory bacterium to be identified. The genus *Bdellovibrio* consists of small, motile gram-negative bacteria that act as predators or parasites of other gram-negative species such as *E. coli* and Pseudomonas. This type of bacterium is a chemoheterotrophic obligate aerobe living optimally in temperatures between 28^{o}C to 30^{o}C. Typical habitats of *Bdellovibrio* include soil, sewage, freshwater, and marine environments. Compared to other bacterial genera *Bdellovibrio* live in relatively dilute concentrations in these natural environments. It can attach to and enter victims, reproduce inside them and destroy the host, producing maximum numbers of *Bdellovibrio* progeny. It was discovered accidentally in 1962 by HEINZ STOLP, who, whilst searching for soil bacteriophages, noticed bacteria attacking Pseudomonas within 3 days of incubation. They were classified in the genus *Bdellovibrio*, which has, since then, increased in the size to include other phenotypically similar organisms.

According to the nutrional criteria, *Bdellovibrio* are divided into three main groups: -

Host-dependent-wild type and predatory, depending on intraperiplasmic growth in susceptible preys, host-independent can multiply in the presence or absence of prey cells, facultative- multiply in the presence of prey cells.

IBRAGIMOV (1980) has been found that cows, horses, pigs and ducks contain *Bdellovibrio bacteriovorus* in their intestine and constantly excrete them with faeces into the enviroment. These microorganisms have not been detected in the faeces of man, white mice, frogs and fish. *Bdellovibrio*, if introduced together with *Shigella* or after them, prevent the development of keratoconjunctivitis in some of the rabbits. No manifestations of the lytic activity of *Bdellovibrio* in relation to *Salmonella* and *Vibrio cholerae* have been observed in the intestine of white mice and young rabbits. (EDAO et al. 1998) have been found *Bdellovibrio* in the faeces of only healthy but not in the diseased Humans

2.5.2 Intercellular morphology

As a free living organism in the environment the *Bdellovibrio* is a flagellated, gram-negative, comma shaped rod that is highly motile. This bacterium is extremely small, having a diameter between 0.5 to 1 micrometers. During this phase *Bdellovibrio* is not growing or reproducing (BURNHAM and CONTI 1984).

2.5.3 Location, attachment, and penetration

Bdellovibrio appear to locate a susceptible host through chance encounter and no observation of chemotaxis has been observed. The bacteria have a high speed collision with its host at the non flagellated site and then it rotates on its axis. Enzymes are then secreted to break down the outer membrane and the cell wall of the gram-negative host. Upon degradation of these structures the *Bdellovibrio* enters the periplasmic space and the host cell forms a spherical structure called a bdelloplast (TUDOR and CONTI 1977).

2.5.4 Growth and production

Immediately upon entrance into the periplasmic space, *Bdellovibrio* loses its flagellum and secretes enzymes that pass into the cytoplasm and degrade the host macromolecules such as proteins, DNA, and RNA. These degradation products are then used by *Bdellovibrio* for synthesis of macromolecules. At this point the host cell has now become an inviable substrate for the predator. *Bdellovibrio* begins elongating to form a helical structure that undergoes multiple fission to produce a maximum of about six daughter cells, depending on the size of the host. The flagellated daughter cells then enter the environment as free living bacteria until they collide with another susceptible host, figure 2.

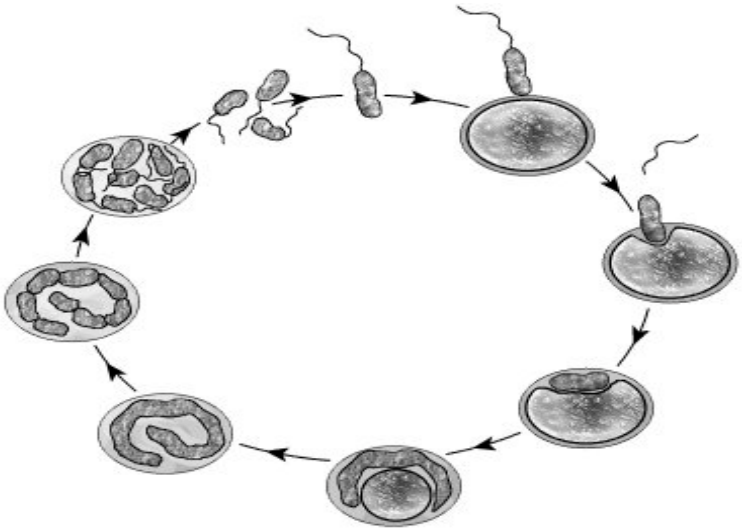


Figure 2. *Bdellovibrio* biphasic life cycle (after RUBY 1992)

They have a non-growing motile predatory phase, and a nonmotile intracellular reproductive phase. In their predatory phase the cells are very motile and chemotactic. The chemotactic behavior is not affected by prey cell density, rather the *Bdellovibrio* migrate toward ionic conditions, which are favored by their particular prey cells. *Bdellovibrio* are motile by means of a single ensheathed polar flagellum and are capable of moving an incredible 100 cell lengths per second. This would allow the cell to traverse a meter in under 2 hours. The reproductive phase of the life cycle begins with the attachment of the *Bdellovibrio* to a prey cell. The *Bdellovibrio* then penetrates the outer membrane and peptidoglycan layer and resides in the periplasmic space of the prey cell. The prey cell loses its

rigidity, and is converted into a spherical bdelloplast. The invading cell will then elongate unidirectionally until it has increased its length by approximately 20 times (or it is limited by prey cell volume). The extension occurs at the non-flagellated end of the *Bdellovibrio*, the flagellated end having been converted into a holdfast type structure. The elongated spiral shaped cell will then undergo concurrent multiple septations and fragment into multiple unit length cells. The bdelloplast is then lysed and the progeny *Bdellovibrio* are released to carry out the predatory phase of their life cycle (RUBY 1992).

2.5.5 Mechanisms of predation

To infect, the *Bdellovibrio* must penetrate both the prey cell outer membrane, and the covalently linked peptidoglycan layer. Mechanical action due to the shear impact of the rapidly moving *Bdellovibrio* may aid in penetration, but the invading cell also has a host of enzymes, which are also necessary. They are:

1) Lipopolysacharidase.
2) Glycanase (rapidly solubalizes 10% of N-acetyl glucosamine during attack).
3) Deacetylase (deacetylates the peptidoglycan, making it immune to further attack by glycanase).
4) Peptidase.

The near instant death of the prey cell and the ability of the *Bdellovibrio* to grow in the periplasmic space have lead researchers to look for mechanisms which would explain this phenomenon. There is a protein found in the cytoplasmic membrane of *Bdellovibrio* infected prey cells, which shows similarity to the outer membrane. It is believed that the *Bdellovibrio* either implants an outer membrane protein of its own into the prey cell's cytoplasmic membrane, or translocates an outer membrane protein belonging to the prey cell into the cytoplasmic membrane. This would result in the instant and complete collapse of the membrane potential, which would kill the cell, and its cytoplasm would be allowed to leak into the periplasm to feed the growing *Bdellovibrio* (TUDOR et al. 1990).

2.5.6 Taxonomy

In order for an organism to be classified as a *Bdellovibrio* it must meet the following criteria:

1) The organism must grow in the periplasmic space of the prey cell.
2) The organism must have ensheathed polar flagella.

The genus is divided into six phage sensitivity groups:

Group 1-4 *Bdellovibrio bacteriovorus*
Group 5 *Bdellovibrio stolpii*
Group 6 *Bdellovibrio starrii*

The taxonomy of *Bdellovibrio* is done in the kind of "grab bag" approach. There are numerous physiological characteristics, which could be used to differentiate the species, but the work has not yet been done (BURNHAM and CONTI 1984).

2.5.7 Ecology and evolution

The low amount of DNA homology between *Bdellovibrio* (0-35%) has led to the suggestion that this group is the result of a variety of organisms convergently evolving a predatory lifestyle. The life cycle requires the accumulation of a particular set of traits. This gives rise to a "form follows function" argument as to why they are so physiologically similar.

It has been determined that *Bdellovibrio* require $1.5x10^5$-10^6 prey cells /ml to sustain themselves. Although it is found in many different environments, it is clear that it could not be uniformly distributed throughout them. It has been shown that sewage pollution can result in prey densities high enough to support *Bdellovibrio* populations. It has also recently been shown that *Bdellovibrio* rapidly colonizes and tenaciously adhere to surfaces in aquatic environments, and may play an important role in the population dynamics of biofilms (BURNHAM and CONTI 1984).

2.5.8 Modern uses

Interestingly enough, some researchers have been looking into the use of *Bdellovibrio* to fight food borne pathogens. Experiments have determined that a moderate dose (5:1 *Bdellovibrio* to prey cells) will lower an *E. coli* population by 90% in one hour. Its efficacy for killing enterics on different sufaces is being explored.

3. Animals, Material and Methods

3.1 Birds and experimental design

Experiment 1:

Thirty one-day-old chicks of layer type naturally infected with *S*. Enteritidis were divided in 3 separate groups. The different groups were reared separately in battery cages. The chicks were reared on a diet free from antibiotics and coccidiostates. A starter-grower ration (FESONI GMK) was provided all the time of the experiment (Bruno Fehse u. Sohn GmbH and Co.). The ration constituents per/kg were as follow; crude protein 20%, raw fats 4,5%, MJ ME/kg 11, methionine 0,4%, calcium 1%, phosphorus 0,6%, vitamin A 10000 IU, vitamin D_3 2000 IU 2000. All birds were provided with ad libitum water and feed. Room light was kept on continuously until the end of experiment. Room temperature was initially set at 32 °C and the temperature was reduced by 2.8 °C the following week. The first group recieved daily 0.5 % inulin of Jerusalem artichoke (J. A.) (I1-group) via drinking water for 3 weeks. The second group recieved the same amount of inulin and food supplemented with 1% linseed daily (IL1-group) for 3 weeks. The third group was the untreated control (C1-group). At 7-day intervals (at 7, 14 and 21th day old) cloacal swabs were taken from all birds and examined for the presence of *Salmonella* positive birds.

Inulin (from LIENIG, Wildfrucht-Verarbeitung GmbH and Co., KG) is characterised by the presence of fructose units (30 fructose units/molecule, molecular weight 5000) with a beta-2, 1-glycosidic bond. It is considered as one of the carbohydrate prebiotic references. Inulin is a fructooligosaccharide (FOS) derived from Jerusalem artichoke. It is water soluble and not digested by monogastric animals. It is not only a natural ingredient but also a prebiotic which stimulates the growth of good intestinal bacteria which support healthy colon conditions.

Composition of Linseed as a food:

(Fat 41%, total dietary fibre 28%, protein 20%, moisture 7% and ash 4%, analysed by the American Oil Chemist's Society). Fatty acid composition of linseed oil [polyunsaturated fatty acids Omega-3 (linolenic acid) 57%, polyunsaturated fatty acids Omega-6 (linoleic acid) 16%, monounsaturated fatty acids (oleic acid) 18% and saturated fatty acids (palmitic and stearic acids) 9%] after BHATTY (1995).

Experiment 2:

Forty-five one-day-old SPF chicks were investigated. The chicks were randomly assigned into three groups. The different groups were reared in separate battery cages under strictly hygienic measures. The chicks were reared on a diet free from antibiotics and coccidiostates. A starter-grower (FESONI GMK) ration was provided all the time of the experiment. All birds were fed ad libitum and had free access to water. Room light was kept on continuously until the end of experiment. Room temperature was initially set at 32 °C and the temperature was reduced by 2.8 °C

the following week. The first group recieved 0.5 % inulin daily (J. A.) via drinking water (I2-group) for 3 weeks. The second group recieved the same amount of inulin and food supplemented with 1% linseed (from BZ Bio-Zentrale GmbH) daily (IL2-group) for 3 weeks. The third group was the untreated control (C2-group). At 7-day intervals (at 7, 14 and 21th day old), 5 chicks from each treatment were weighted and euthanisied. The BF were collected and weighted. Food and faecal samples were aseptically collected from the crop, small intestine, caecum and rectum for studying the changes in total, gram-negative and *Bdellovirio* bacterial counts.

Experiment 3:

Two broiler farms (5000 birds per farm) were given 0.5% inulin (J. A.) via drinking water daily for 5-6 weeks starting from the first day of age and each farm was divided into experimental and control groups:

1. Farm I M., herd 75 with inulin and herd 76 as control.
2. Farm II M., herd 251 with inulin and herd 165 as control.

All birds were reared on a diet free from antibiotics and coccidiostates. The contents of the diet were as follow: 40% corn 27.5% soja, 15%wheat, 7% fat, 6% raps extraction pellet, 2% peas, 0.75% lime, 0.75% dicalciumphosphate, 1% vitamin-trace elements mixture.

Five birds were randomly selected from each group per week. These birds were weighted and euthanisied:

1- Caecal samples were collected aseptically for bacteriological examinations.
2- Blood samples (serum) for endotoxin, PC-BP examinations.
3- Bursa of Fabricious and pancreas were collected and weighted.

Experiment 4:

1-Four (R. I, R. II, Ro., and Co.) different broiler farms (5000 birds per farm)

Caecal, blood samples, BF and pancreas were collected aseptically from 5 birds weekly (during 4 weeks) for analysis of caecal flora and studying its relation with endotoxin, PC-BP, P/BW and BF/BW ratios. These birds were not administered with inulin and not supplemented with linseed.

2-Two (Ho. – Wa.) breeder farms (5000 birds per farm)

Caecal, blood samples were collected aseptically from 5 birds (2 weeks interval during 10 weeks) for analysis of caecal flora and studying its relation with endotoxin, PC-BP. These birds were not administred with inulin and not supplemented with linseed.

3.2 Bacteriological Examinations

Food or faecal samples of crop, small intestine, caecum, rectum and cloaca were collected under aseptic condition for bacteriological investigations. One-gram of the sample from each chick was weighted and placed in a sterile glass tube. The initial dilution was made by mixing one-gram of the sample with 9 ml of sterile HM buffer solution. 30 µl from the suspension was then withdrawn and further diluted in sterile microplates in 10-fold dilutions. From each of the serial dilution, 10 µl of the suspension was taken and plated on the growth media for quantitative and qualitative identification of bacteria. Nutrient agar (Difco) was used for total aerobic bacterial count (figure 3), MacConkey agar (Difco) for gram-negative bacterial count (figure 4). The petri-dishes were incubated aerobically at 37°C for 24 hours and neomycin-polymyxin blood agar and egg yolk agar for *C. perfringens* (incubated anaerobically at 37°C for 48 hours), and double layer agar (incubated aerobically at 30°C for 2-7 days) for *Bdellovibrio*. For isolation and identification of *S.* Enteriditis, swabs of cloacal faeces (approximately 1 g) were pre-enriched separately in 9 ml of buffered peptone water incubated at 37°C for 24 hours. About 0.15 ml of the broth was transferred onto diagnostic selective einrichment in Rappaport Vassiliadis-broth, incubated at 41.5°C for 24 hours and then subcultured onto xylose lysine desoxycholate (XLD, Oxoid). XLD was incubated at 37°C for 24 hours and suspected *Salmonella* colonies identified serologically using slide agglutination tests.

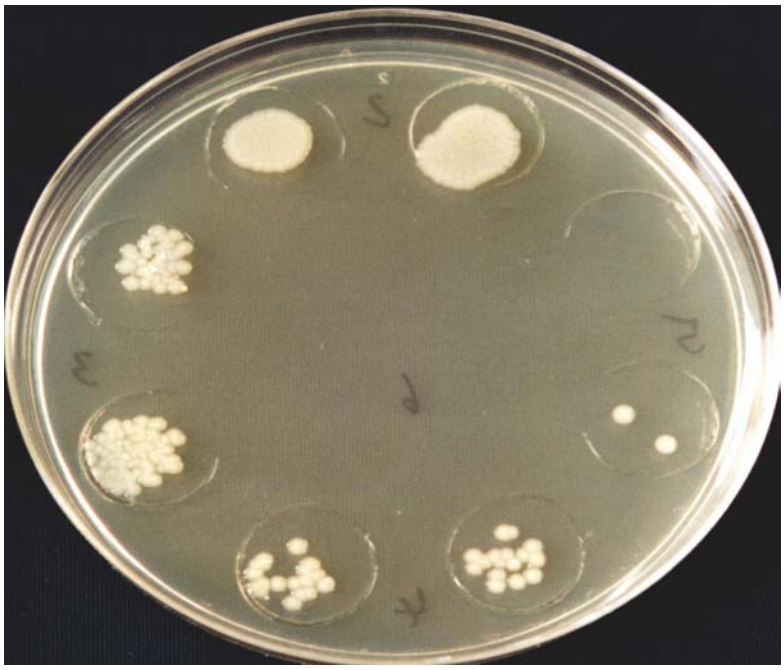


Figure 3. Quantitative identification of total aerobic bacterial count on nutrient agar, 24 h, 37 ºC. Magnification x 0.4

Figure 4. Quantitative identification of gram-negative bacterial count on MacConkey agar, 24 h, 37 ºC. Magnification x 0.4

***C. perfringens* identification:-** On neomycin-polymyxin blood agar (sheep blood) and egg yolk lactose agar, incubated anaerobically in a sealed jar (Oxoid) at 37 °C for 48 hours, *C. perfringens* is indicated by circular, smooth colonies 2-4 mm in diameter, surrounded by an inner zone of complete haemolysis and outer zone of discoloration and incomplete haemolysis (double zone haemolysis, figure 5). Growth on egg-yolk lactose agar demonstrates the presence of lecithinase and phosphatase activity (SCHALLEHN and BRANDIS 1973), using phosphatase reagent, *C. perfringens* colonies appeared as dark brown colonies) and absence of lipase. Also *C. perfringens* ferments lactose, figure 6.

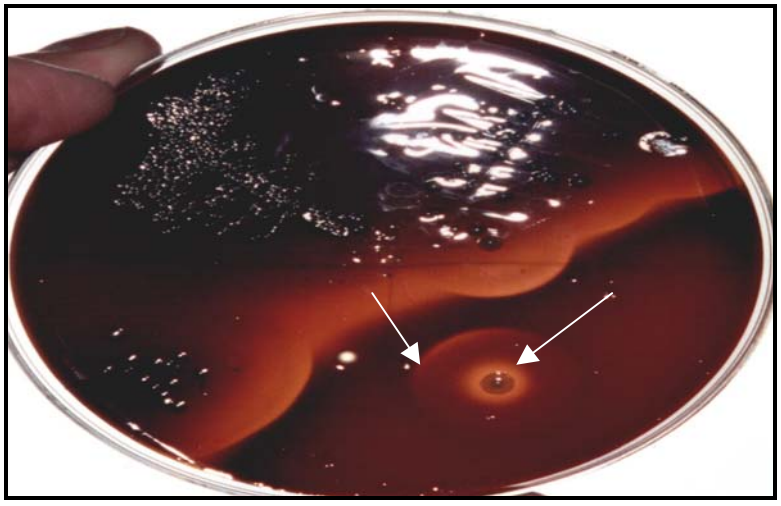


Figure 5. *C. perfringens* is indicated by circular, smooth colonies 2-4 mm in diameter, surrounded by an inner zone of complete haemolysis and outer zone of discoloration and incomplete haemolysis (double zone haemolysis), 48 h, 37 ºC. Magnification x 0.4

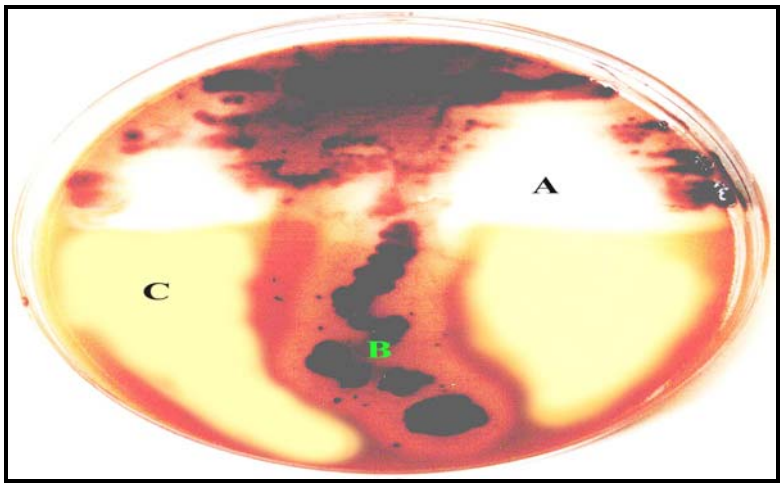


Figure 6. Growth of *C. perfringens* on egg-yolk lactose agar demonstrates the presence of lecithinase (A) and phosphatase (B) and fermentation of lactose (C), 48 h, 37 ºC. Magnification x 0.4

C. perfringens are gram-positive to gram-variable, which appear as plump rods with blunt ends, figure 7.

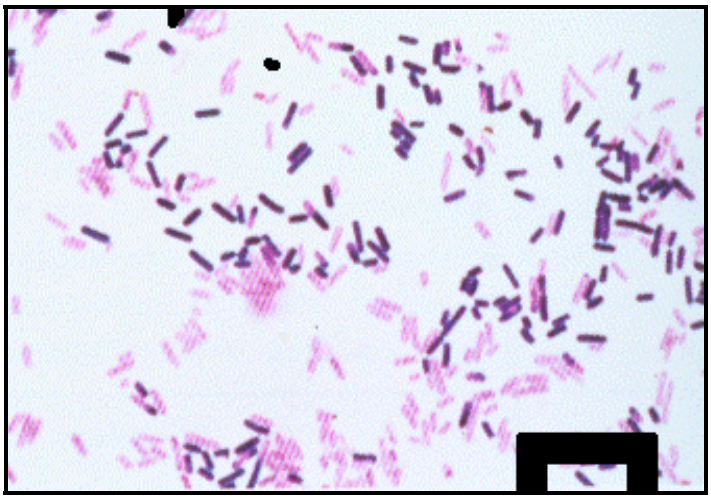


Figure 7. *C. perfringens*, gram stain, gram-positive to gram-variable, which appear as plump rods with blunt ends. Magnification x 1000

Isolation and identification of *Bdellovibrio* spp.

Double-layer technique

The blocking layer agar consists of bottom and top layer agar. The trichloroethylene YP medium was added in order to promote the activity of the *Bdellovibrio*, in bottom and top layer agar (STOLP and PETZOLD 1962). Composition of the trichloroethylene YP medium: yeast extract (Difco) 3.0 g of peptone (Difco) 0.6 g trichloroethylene (Tris) buffer (50 mM, pH 7.5) 1000 ml.

Preparing growth media-bottom agar

Manufactured (after SEIDLER and STARR 1969) as follows: - bottom layer agar, 12g agar (Difco) was added to 1000 ml distilled water which contains 3 mM $MgCl_2$ x 6 H_2O and 2mM $CaCl_2$x 2 H_2O and autoclaved at 121°C for 15 min. After cooling at 60 °C, 100 ml sterile trichloroethylene YP medium was added into the agar. The petri dishes were kept under sterile conditions 2-3 days at ambient temperature before used.

Preparing growth media-top agar

6 g agar (Difco) was added to 1000 ml distilled water, which contains 3 mM $MgCl_2$ X 6 H_2O and 2mM $CaCl_2$ x 2 H_2O after that autoclavering at 121°C for 15 min. After cooling at 60 °C, 100 ml sterile trichloroethylene YP medium was added into the agar and dispersed in 5 ml/ sterile tube.

Preparing growth media for prey cells (*E. coli*, K 12)

The growth media constituents were as follow, 1000 ml distilled water, 20 g glucose, 10 g yeast extract, 20 g $CaCO_3$ and 15 g agar. The pH was adjusted to 7.6 and then all constituents were mixed thoroughly and autoclaved at 121°C for 15 min. after that it was dispersed as a thin-layer over several petri dishes.

Composition of the HM buffers (after TUDOR and CONTI 1977)

The HM buffer was manufactured by addition of 1mM $CaCl_2$ (111mg) and 0, 1 mM $MgCl_2$ (9.5 mg) for the Hepes buffer (N-2 hydroxyethyl piperazin-N-2 ethanosulfonic acid) of the company Sigma as 10 mM solution (2.38 g in 1000ml dist. Water) , pH was adjusted to 7.6 and autoclaved at 121°C for 15 min.

Procedure of *Bdellovibrio* examination in caecal sample

1 g of caecal sample was suspended in 9 ml sterile HM buffer, and mixed vigorously and diluted in 10-er steps. From each dilution 0.5 ml caecal sample was mixed thoroughly with 1ml prey cells (10^9/ml concentration) in the top layer agar, which poured on the bottom agar and allows cooling until solid (pouring method). The prey cells can mixed with the top agar and poured on the bottom agar and allow cooling until solid and then it can 0.5 ml caecal samples from each dilution dripped on the 2 layers agar (dropping method). The plates were incubated at 30-37°C for 2-7 days after that the plates could be checked for the presence of plaques which are possible to be *Bdellovibrio* colonies, figure 8 and 9. To verify isolation of *Bdellovibrio* colonies, these colony areas were examined onto wet mount slides to viewed under a phase contrast microscope and looking for tiny, motile, comma shaped organisms (0.25-0.4 micrometers in width) attached to *E.coli* K12 (prey cells), figure 10 and 11. By conducting a gram stain test. Both *E.coli* and *Bdellovibrio* are gram-negative (pink), figure 12.

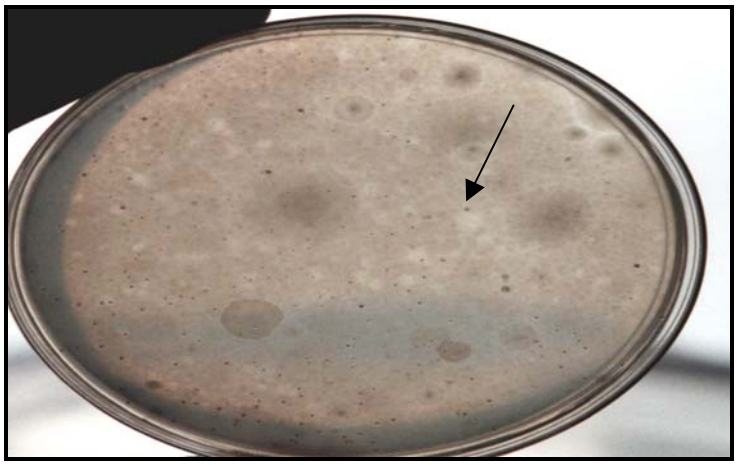


Figure 8. Four-day-old plaque colonies (arrow) of *Bdellovibrio bacteriovorus* on a lawn of *E. coli* K12 (pouring method)

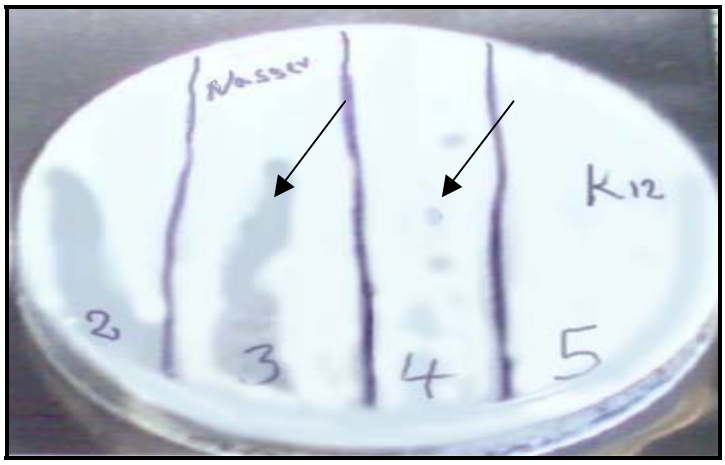


Figure 9. Four-day-old plaque colonies (arrow) of *Bdellovibrio bacteriovorus* on a lawn of *E. coli* K12 (dropping method)

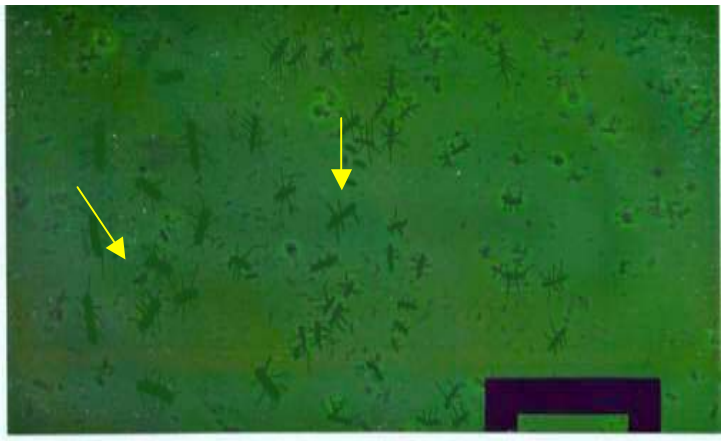


Figure 10. Under a phase contrast microscope, looking for tiny, motile, comma shaped organisms (0.25-0.4 micrometers in width) attached to *E. coli* K12 (prey cells)

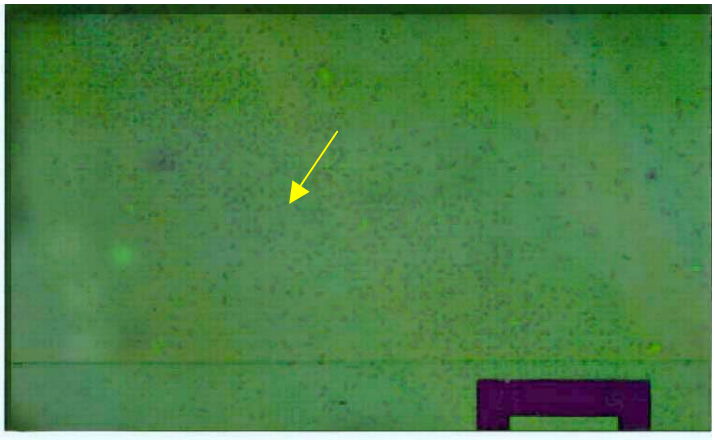


Figure 11. Under a phase contrast microscope, looking for tiny, motile, comma shaped organisms (0.25-0.4 micrometers in width)

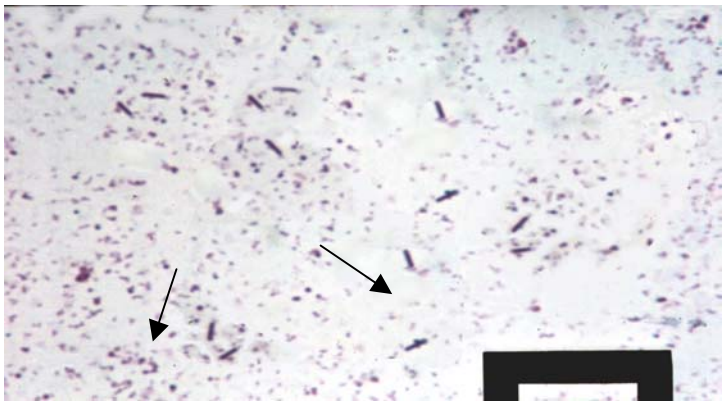


Figure 12. Gram stain test, both *E. coli* (right arrow) and *Bdellovibrio* (left arrow) is gram-negative (pink colour)

3.3 Determination of endotoxin level in serum

3.3.1 Quantitive chromogenic LAL (Limulus-Amoebocyte-Lysate) Test:

LAL test is a quantitative test for the detection of gram-negative bacterial endotoxin. A serum sample was mixed with the LAL supplied in the test kit and incubated at 37°C for ten (10) min. A substrate solution was then mixed with the LAL-sample and incubated at 37°C for an additional six (6) min. The reaction was stopped with stop reagent. If endotoxin was present in the serum sample, a yellow colour will develop. The absorbance of the serum sample can be determined spectrophotometrically at 405-410 nm. Since this absorbance is in direct proportion to the amount of endotoxin present, the concentration of endotoxin in unknown serum samples can be calculated from a Standard curve. The use of LAL for the detection of endotoxin evolved from the observation by BANG (1956) that a gram-negative infection of Limulus Polyphemus, the horse-shoe crab, resulted in fatal intravascular coagulation. LEVIN and BANG (1964a, b) later demonstrated that this clotting was the result of a reaction between endotoxin and a clottable protein in the circulating Amoebocytes of Limulus. LEVIN and BANG (1968) prepared a lysate from washed Amoebocytes

which was an extremely sensitive indicator of the presence of endotoxin. YOUNG et al. (1972) have purified and characterized the clottable protein from LAL and have shown the reaction with endotoxin to be enzymatic.

3.3.2 Principle of the LAL Test

The present LAL method utilizes the initial part of the LAL endotoxin reaction to activate an enzyme, which in turn releases p-nitroaniline from a synthetic Substrate, producing a yellow colour.

1. Proenzyme $\xrightarrow{\text{Endotoxin}}$ Enzyme
2. Proenzyme $+H_2O$ $\xrightarrow{\text{Enzyme}}$ Peptide + pNA

Gram-negative bacterial endotoxin catalyzes the activation of a proenzyme in the Limulus Amoebocyte Lysate (LAL). The initial rate of activation is determined by the concentration of endotoxin present. The activated enzyme catalyzes the Splitting of p-nitroaniline (pNA) from the colourless Substrate Ac-lle-Glu-Ala-Arg-pNA. The pNA release is measured photometrically, at 405-410 nm after the reaction was stopped with stop reagent. The correlation between the absorbance and the endotoxin concentration is linear in the 0.1-1.0 EU/ml range. The concentration of endotoxin in a serum sample is calculated from the absorbance values of solutions containing known amounts of endotoxin standard.

3.3.3 Reagents:

All reagents supplied from BIO•WHITTAKER, A CAMREX Company.

3.3.3.1 *E. coli* Endotoxin - Stored at 2-8°C

Two vials containing approximately 15-30 EU lyophilized endotoxin which reconstituted by adding I.0 ml of LAL reagent water warmed to room temperature. The actual concentration of the vial will be determined by the value stated on the enclosed certificate of analysis. For example, if the value of the vial is 24 EU, when reconstituted with 1.0 ml water it will yield a concentration of 24 EU/ml. The vial must be shaked vigorously for at least 15 minutes, preferably with a vortex mixer. This stock solution is stable for one month at 2-8 °C. Prior to use, the solution must be warmed to room temperature and vigorously mixed for 15 minutes. This is important because the endotoxin tends to attach to glass.

Other endotoxin preparations may be used to prepare the standards, howover, and their performance in the chromogenic assay relative to the Reference Standard Endotoxin (RSE) must be determined.

3.3.3.2 Chromogenic substrate – Stored at 2-8°C

Five vials containing approximately 7 mg lyophilized substrate. Which can be reconstituted by adding 6.5 ml or LAL reagent water to yield a concentration of 2mM. Once reconstituted, the substrate solution is stable for one month at 2-8°C, if not contaminated with microorganisms or pyrogens. The substrate must be protected from long-term exposure to light.

3.3.3.3 Limulus Amoebocyte Lysate (LAL) Stored at 2-8°C

Five vials containing lyophilized lysate prepared from the circulating Amoebocytes of the horseshoe crab Limulus Polyphemus. Which must be reconstituted immediately before used with 3.0 ml LAL Reagent Water. If the contents of more than one vial are required. Two or more vials can be pooled before use. To avoid foaming, it must be swirled gently. Reconstituted lysate can be stored at -10°C or colder up to one week if frozen immediately after reconstitution. After that it can be thawed and used only once.

3.3.4 Specimen collection and preparation

All materials coming in contact with the specimen or test reagents must be endotoxin-free. Materials may be rendered endotoxin-free by heating at 250°C for 30 minutes. Appropriate precautions should be taken to protect depyrogenated materials from subsequent environmental contamination. It may be necessary to adjust the pH of the serum sample to within the range 7.0-8.0 using endotoxin-free sodium hydroxide or hydrochloric acid. The pH of an aliquot of the bulk sample must be always measured to avoid contamination by the pH electrode. Some compounds may induce or give false positive or false negative results i.e. blood products (like serum), polynucleotides, solutions containing heavy metals, or those of high ionic strength or osmolarity FRIBERGER et al. (1982) have reported the removal of non specific inhibition in blood products by diluting the test sample 1:10 in LAL reagent water and heating at 70°C for five minutes. Serum samples to be tested must be stored in such a way that all bacteriological activity is stopped or the endotoxin level may increase with time. For example, the serum samples must be stord at 2-8°C for less than 24 hours, samples stored longer than 24 hours should be frozen.

3.3.5 Preliminary reagent preparation

In each series of determination, four standard endotoxin solutions should to use. The table (7) below suggests a dilution scheme for the construction of these standards from the endotoxin supplied in the kit. Alternative dilution schemes can be used as well as other endotoxins not supplied in this kit. The initial dilution from the endotoxin stock is 1/X. where X equals the concentration of the endotoxin vial. This yields an endotoxin solution containing 1.0 EU/ml For example, if the potency is 23 EU/ml, the initial dilution is 1/23 or 0.1 ml of endotoxin stock into 2.2 ml of LAL reagent water.

Table 7. A dilution scheme for the construction of these standards from the endotoxin supplied in the kit

Endotoxin Concentrations EU/ml	Endotoxin Stock Solution	Endotoxin Standard Solutions 1 EU/ml	LAL Reagent Water
1.00	0.1 ml		(x-1) ml/10
0.50		0.5 ml	0.5 ml
0.25		0.5 ml	1.5 ml
0.10		0.1 ml	0.9 ml

x = endotoxin concentration of the vial

1- A solution containing 1.0 EU/ml endotoxin can be prepared by diluting 0.1 ml of the endotoxin stock solution with (x-1)/10 ml of LAL reagent water in a suitable container, where x equals the endotoxin concentrations of the vial. This solution should be vigorously vortexed for at least 1 minute before proceeding. For example, if x = 23 EU/ml, then 0.1 ml of the endotoxin stock solution can be diluted with 2.2 ml, (23-1)/10, LAL reagent water.

2- Afterthat, 0.5 ml of the 1.0 EU/ml solutions can be transferred into 0.5 ml of LAL reagent water in a suitable container and labelled as 0.5 EU/ml. This solution should be vigorously vortexed for at least 1minute before use.

3- 0.5 ml of the 1.0 EU/ml solutions can be transferred into 1.5 ml of LAL reagent water in a suitable container and label 0.25 EU/ml. This solution should be vigorously vortexed for at least 1 minute before use.

4- 0.1 ml of the 1.0 EU/ml solutions can be transferred into 0.9 ml of LAL reagent water in a suitable container and labelled as 0.1 EU/ml. This solution should be vigorously vortexed for at least 1 minute before use.

3.3.6 Test procedure

The addition of all reagents in the limulus assay must be consistent. All microplates' wells must be treated in exactly the same manner in order to determine the proper endotoxin concentration. It is suggested that, in a series of tests, reagents should be pipetted in the same order from well to well, and at the same rate, table 8.

Table 8. The outlines of the test procedure for endotoxin examination

	Sample	Blank
Test (serum)sample or standard at 20-25°C	50 µl	
LAL reagent water		50 µl
LAL	50 µl	50 µl
Mixing and incubation at 37°C ± 1.0 °C	10 min	10 min
Substrate solution at 37°C ± 1.0 °C	100 µl	100 µl
Mixing and incubation at 37°C ± 1.0 °C	6 min	6 min
Stop reagent	100 µl	100 µl
Mix immediately		

3.3.6.1 Microplate method

1. Pre-equilibrate the microplate at 37°C ± 1.0 °C in the heating block adapter.

2. While leaving the microplate at 37°C ± 1.0 °C, 50 µl of serum sample or standard can be carefully dispensed into the appropriate microplate well. Each series of determinations must include a blank plus the four endotoxin standards run in duplicate. The blank wells contain 50 µl of LAL reagent water instead of sample. All reagent additions and incubation times are identical.

3. At time T = 0.50 µl of LAL can be added to the first microplate well, or first column of microplate wells if using a multi-channel pipettor and reagent reservoir. The timing beginn as the LAL is added. It is important to be consistent in the order of reagent addition from well to well, and in the rate of pipetting. Once the LAL has been dispensed into all microplate wells containing serum samples or standards, then the microplate will be removed from the heating block adaptor and the side of the plate must be tapped to facilitate mixing. Then the plate must return to the heating block adaptor and the cover will be replaced.

4- At T =10 minutes, 100 µl of substrate solution can be added (prewarmed to 37°C ± 1.0 °C). The substrate solution can be pipetted in the same manner as In Step 3. A consistent pipetting rate must be maintained. Once the substrate solution has been dispensed in all microplate wells, the microplate can be briefly removed from the heating block adaptor and repeatedly the side of the plate must be tapped to facilitate mixing. Then the plate must return to the heating block adaptor and the cover will be replaced.

5- At T=16 minutes, 100 µl of stop reagent can be added. The same pipetting order maintained as in Steps 3 and 4. Once the stop reagent has been dispensed into all microplate wells, the plate can be removed and and repeatedly the side of the plate must be tapped.

6- The absorbance of each microplate well can be read at 405--410 nm. By using distelled water the photometer must be adjusted to zero absorbance. The performance characteristics of certain rnicroplate readers are optimal with sample volumes less than 300 µl. The final reaction volume per well can be reduced by adding only 50 µl of the above suggested stop reagents without adversely affecting the test results.

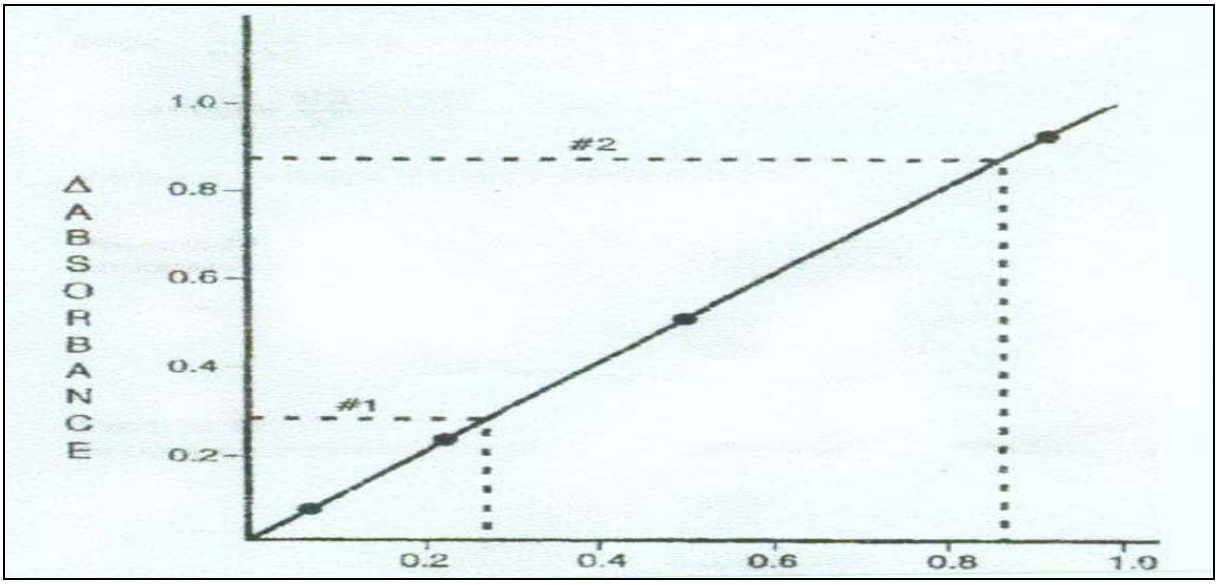
.3.7 Calculation of endotoxin concentration

Under the standard conditions, the absorbance at 405-410 nm is linear in the concentration range of 0.1 to 1.0 EU/ml endotoxin. There are several methods to determine the endotoxin concentration of serum samples. Subtract the mean absorbance of the blank from the mean absorbance value of the standards and serum samples to calculate mean Δ absorbance.

3.3.7.1 Graphic method

Plot the mean Δ absorbance for the four standards on the y-axis and the corresponding endotoxin concentration in EU/ml on the x-axis. Draw a best fit straight line between these points and determine endotoxin concentrations of samples graphically, figure 13.

Figure 13. Determination of endotoxin concentration using the graphic method



Endotoxin concentration (EU/ml), # 1= 0,32 EU/ml, # 2=0,87 EU/ml

3.3.7.2 Calculator method

A calculator equipped with linear regression capability can be used. Enter the mean Δ absorbance and the corresponding concentrations of the four standards. Determine the corresponding endotoxin concentration of the serum samples from their absorbance by linear regression.

Slope = (sy/sx) r

Y-intercept = $\sum$ y/N-($\sum$x/N • slope)

$$r = \frac{N\sum xy - (\sum x)(\sum y)}{N\,(N\text{-}1\ SxSy)}$$

$$\text{Endotoxin concentration} = \frac{\Delta\ \text{Absorbance} - \text{y. intercept}}{\text{Slope}}$$

x = Endotoxin concentration in EU/ml

y = Mean Δ Absorbance value

∑x = Summation of concentration of standards used in EU/ml

∑y = Summation of Mean Δ Absorbance values

∑xy = Summation of the standard concentrations times Mean Δ Absorbance values

Sx = Stanard deviation of x

Sy = Stanard deviation of y

3.4 Determination of phosphoryl choline-binding protein (PC-BP) with enzyme-immuno-assay after (SCHRÖDL et al. 1995, 1998)

3.4.1 Reagents

1. PC-BSA conjugate --→ phosphoryl choline bounded to bovine serum albumin (BSA). The stock solution (40 mg BSA + 1 µg PC-BSA conjugate), from this solution 1/40000 in carbonate-bicarbonate buffer 0.1 M, pH 9.6 and incubated for one hour at room temperature.
2. Wash-buffer (PBST) ----→ PBS pH 7.4 + 0.1% Tween 20
3. Assay buffer----→-20 mM Tris (pH 8.0), + 0.9% NaCl, 0.1% BSA, 0.1% Tween 20 and 5mMCaCl2
4. Avidin-peroxidase (stock solution 1mg/ml diluted in assay-buffer 1/10000).
5. Peroxidase substrate-----→ Tetramethyl-benzidin 10.25 mg/ml in 0.2 M citrate-buffer (pH 4.0+0.015% H_2O_2.

3.4.2 Test procedure

1. 100 µl of PC-BSA conjugate were dispensed per well of the microplate (F-form -96 wells) and incubated at room temperature for 1 hour. The wells washed 2 times with PBST buffer.
2. A valid standard curve with human CRP in the following concentration (2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125 and 0 µg /ml was used to calculate the PC-BP concentrations in serum samples.
3. The serum samples were diluted with PBS 1/50 and 50 µl of the samples were dispensed with multichannel per well.
4. After that, 50 µl Biotin-human C-reactive protein (stock solution 1mg/ml diluted in assay-buffer (1/10000) was dispensed per well and the plate was incubated at room temperature for 1 hour.
5. The wells were washed 3 times with PBST-buffer and 100 µl of diluted avidin-peroxidase was dispensed per well.
6. The plate was incubated at room temperature for 1 hour after that, the wells washed 3 times with PBST-buffer.
7. 100 µl of peroxidase substrate was dispensed per well and the plate was incubated at room temperature for 15 minutes.
8. 50 µl 1M H_2SO_4 was dispensed per well and after that measurement the optical density with 450 nm.

Definition: - 1 Relative Unit = 1µg/ml human CRP

3.5 Lymphoid organs weights

Chickens were weighed prior to euthanasia. The BF and P were removed and weighted. The organ weight measured to the nearest mg and expressed as the percentage of body weight as BF/ BW and P/BW ratios.

3.6 Statistical analysis

Biostatistical analysis: - the available results were statistically processed with the statistical program SPSS 10. The values of the total aerobic bacterial count, gram-negative, *Bdellovibrio*, *C. perfringens* was logarithmed, in order to receive a normal distribution of the values. The examination on normal distribution of the values was accomplished with the Shapiro wilk test. From the logarithm's values of of the total aerobic bacterial count, gram-negative, *Bdellovibrio*, *C. perfringens* and from the values of BW, BF, P, BF/BW and P/BW ratios the average values and the standard deviation (±S) were calculated. The significance examinations with the parameter-free U-test after MAN-WHITNEY were accomplished. The correlation computations took place with the coefficient of correlation (r_s) after SPEARMAN.

4. Results

4.1 Effect of inulin (0.5% via drinking water) and feed supplemented with 1% linseed on the naturally colonization of young chicks with *S.* Enteritidis (Experiment 1)

The addition of inulin to the drinking water (I-group) or inulin and linseed to the young chicks (IL-group) reduced the incidence of a naturally intestinal colonization with *S.* Enteritidis. One week after inulin and linseed applications, the *Salmonella* colonization of the gastrointestinal tract was reduced from 100% to 80% in the I-group and to 60% in the IL-group whereas there were no changes in the *Salmonella* colonization in control animals (C-group). In the second week of the experiment the *Salmonella* colonization was 80%, 60% and 40% in C, I and IL-groups respectively. In the third week of the experiment both I and IL-groups (3, 4) were negative to *Salmonella* colonization and in C-group the colonization was reduced to 40%, table 9, figure 14.

Table 9. Effect of water- administered inulin (0.5%) and feed supplement with 1% linseed on the naturally colonization of young chicks with *S.* Enteritidis

Age of birds in days	* Number of *Salmonella* positive chick/ Number of examined birds			Colonization index [1]		
	C[2]	I-group[3]	IL-group.[4]	C	I-group	IL-group
1	10/10	10/10	10/10	100	100	100
7	10/10	8/10	6/10	100	80	60
14	8/10	6/10	4/10	80	60	40
21	4/10	0/10	0/10	40	0	0

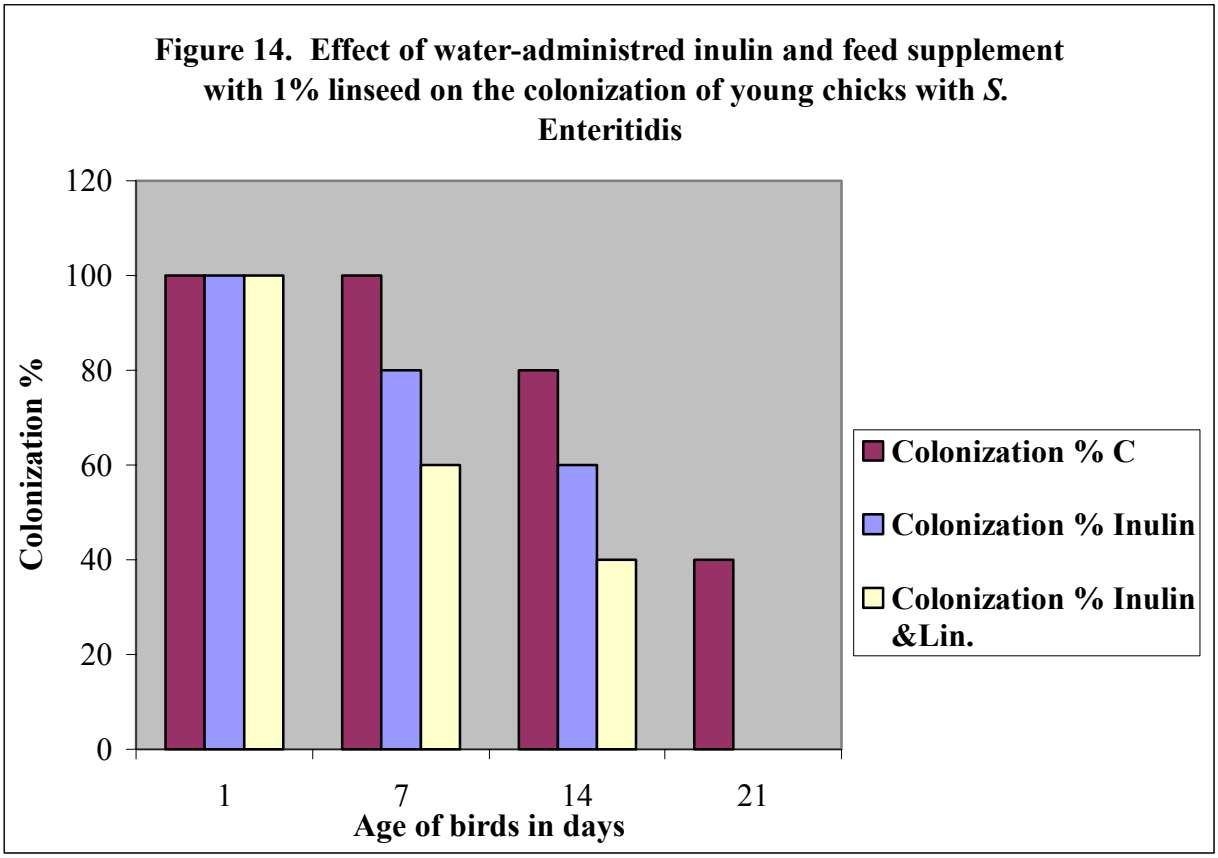
*Cloacal samples were taken from all birds of 3 different groups for bacteriological examination

1= Colonization index (%) means number of *Salmonella* positive chicks divided by the total number of chicks X 100.

2= C means control birds.

3= I (inulin) group, which recieved 0.5% inulin via drinking water.

4= I L- group, which recieved 0.5% inulin via drinking water and feed supplemented with 1% linseed.



4.2 Effect of inulin (0.5 % via drinking water) and feed supplemented with 1% linseed on the intestinal bacterial population and the immune status of SPF chickens (Experiment II)

The bacteriological examinations revealed that there was a significant decrease in total aerobic bacterial count (p=0.049 – 0.005) in the small intestine and caecum (p=0.007 – 0.01) only but not in the crop and rectum of the IL-group and I-group respectively in the third week of the trial (figures 15, 17, 19 and 22) in comparison with control birds, table (1A, 2A, 3A and 4A, appendix). Gram-negative bacterial counts were also significantly decreased in crop (p=0.03-0.04) and small intestine (p=0.003-0.027) of IL-group and I-group at the second week and in the caecum (p=0.03-0.05) and rectum (p=0.02-0.04) at the third week but not in the C-group (figures 16, 18, 20 and 23 and table (1B, 2B, 3B and 4B, appendix).

Bdellovibrio bacteriovorus counts were significantly increased only in the caecum (p=0.011-0.021) and rectum (p=0.012-0.045) of the IL-group and I-group respectively in the second week but can not be isolated from the C-group (figures 21 and 24 and table 3B, 4B, appendix).

There was no significant difference in body weight among all groups but there was a significant increase in BF/BW and P/ BW ratios ($p \leq 0.049$) in the I- group and ($p \leq 0.001$) in the IL-group than in C-group (figures 25 and 26) indicated increased immune capacity of IL-group and I-group.

Figure 15. Changes in total aerobic bacterial counts in the crop of I-group, IL-group and C-group of SPF chickens

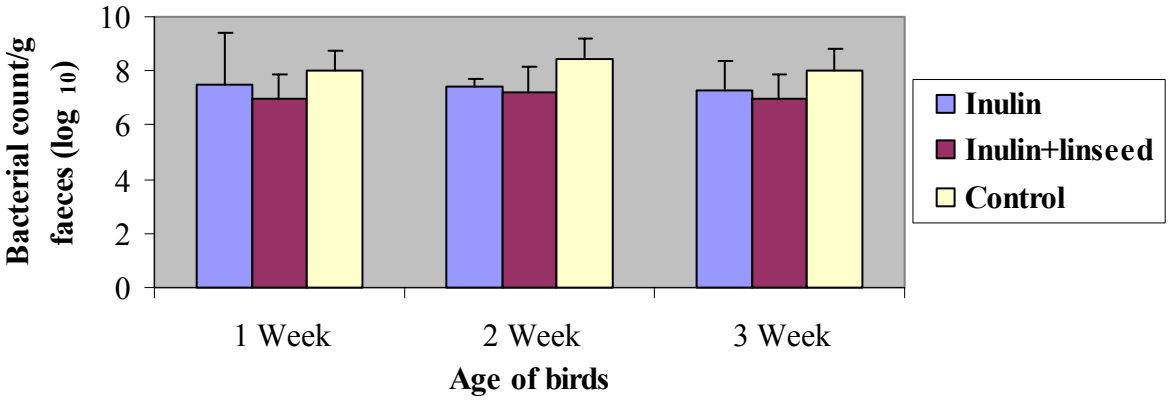


Figure 16. Changes in gram-negative bacterial counts in the crop of I-group, IL-group and C-group of SPF chickens

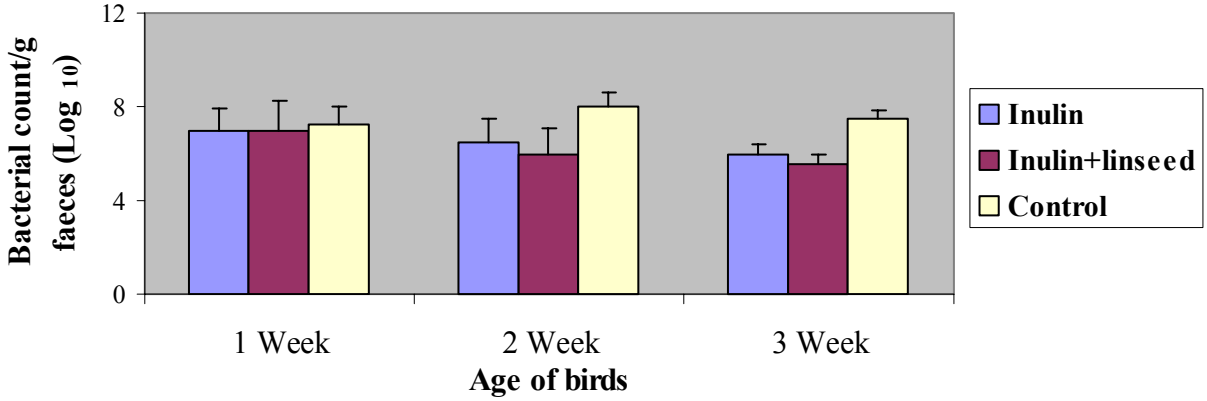


Figure 17. Changes in total aerobic bacterial counts in the small intestine of I-group , IL-group and C-group of SPF chickens

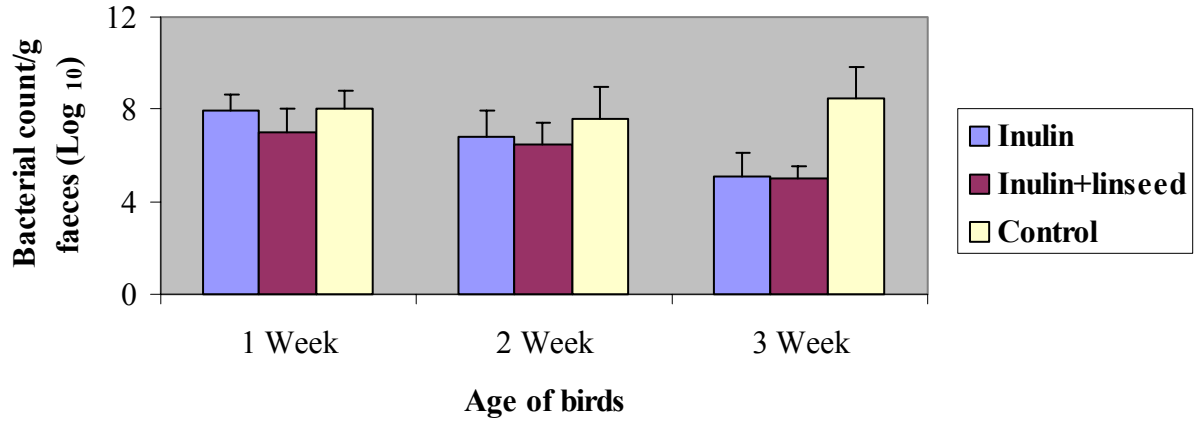


Figure 18. Changes in gram-negative bacterial counts in the small intestine of I-group , IL-group and C-group of SPF chickens

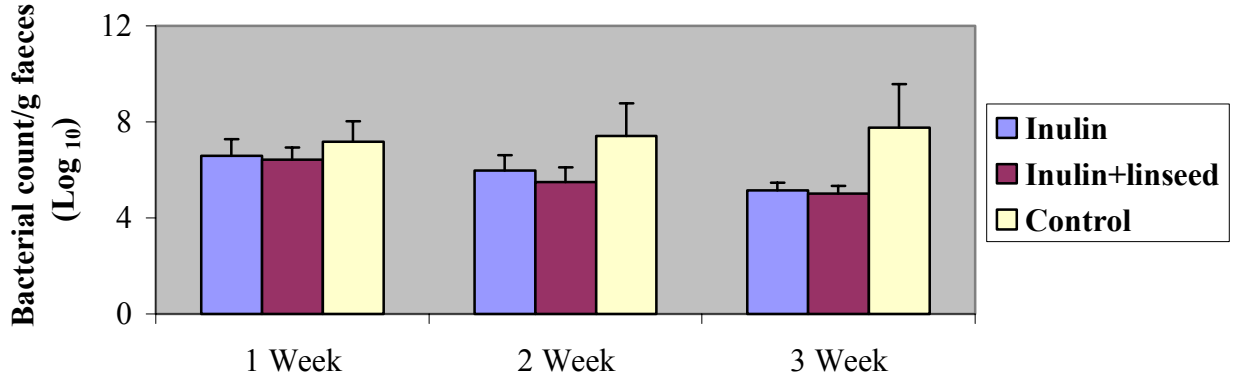


Figure 19. Changes in total aerobic bacterial counts in the caecum of I-group , IL-group and C-group of SPF chickens

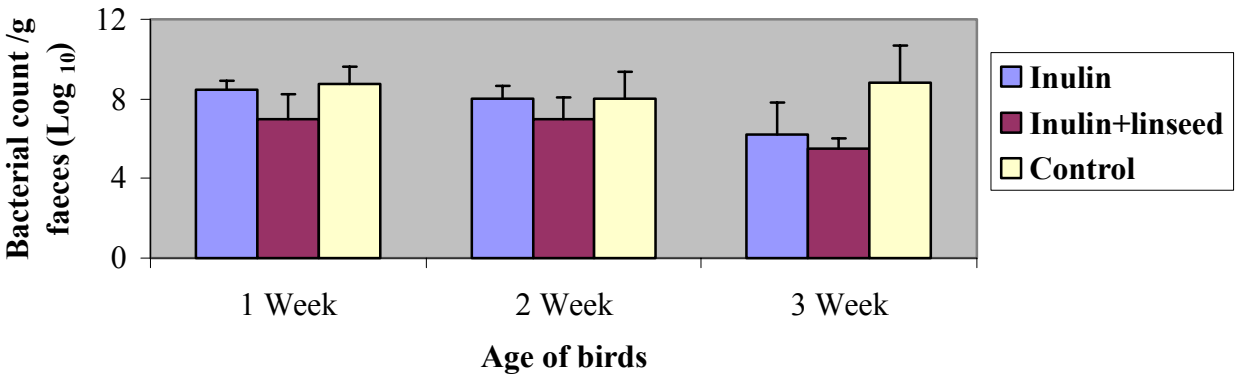


Figure 20. Changes in gram-negative bacterial counts in the caecum of I-group , IL-group and C-group of SPF chickens

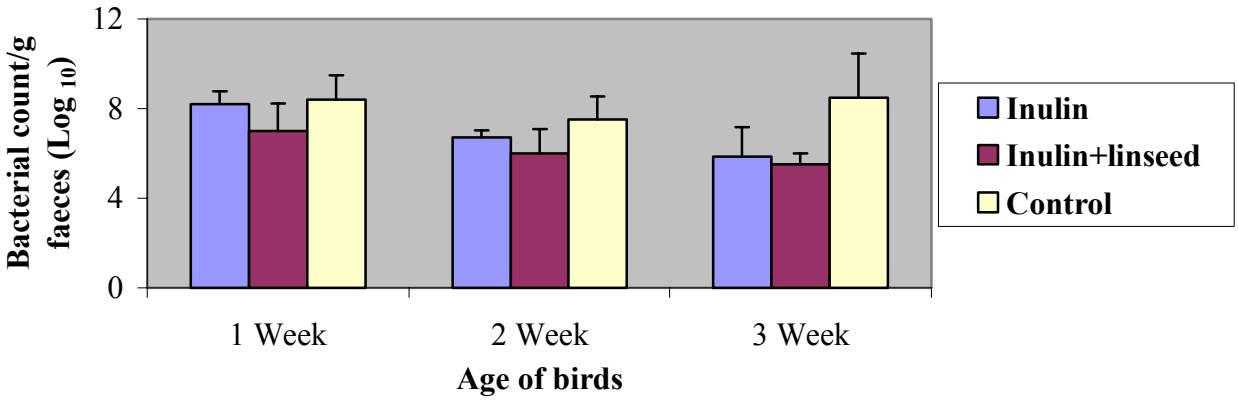


Figure 21. Changes in *Bdellovibrio* counts in the caecum of I-group , IL-group and C-group of SPF chickens

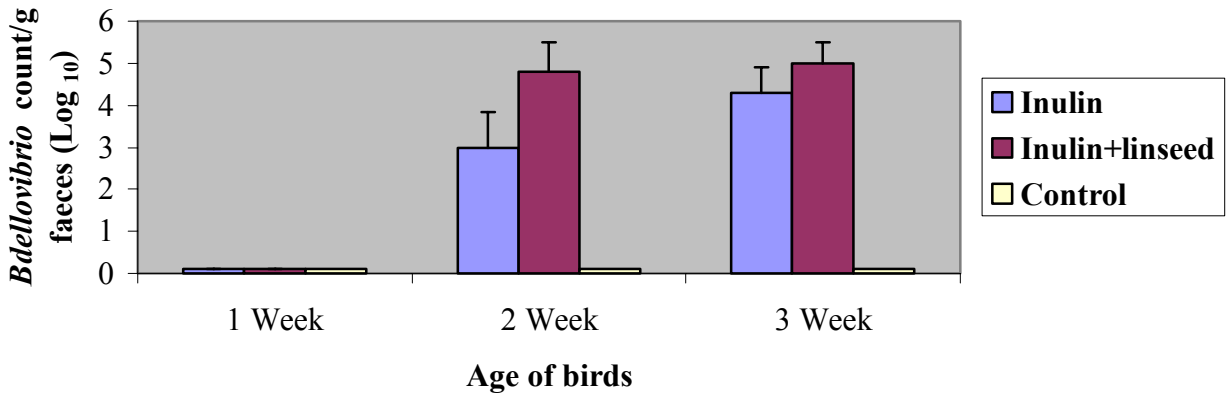


Figure 22. Changes in total aerobic bacterial counts in the rectum of I-group , IL-group and C-group of SPF chickens

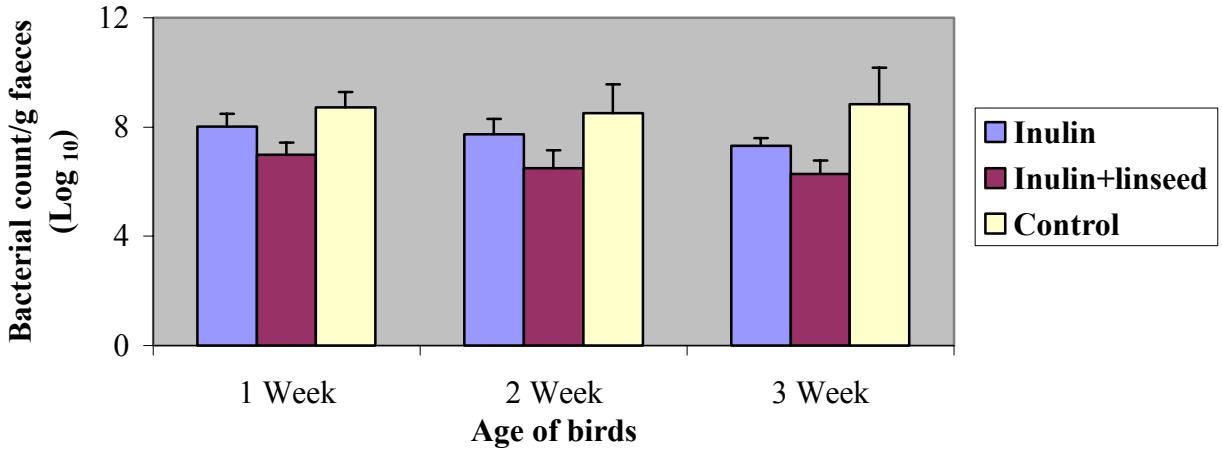


Figure 23. Changes in gram-negative bacterial counts in the rectum of I-group , IL-group and C-group of SPF chickens

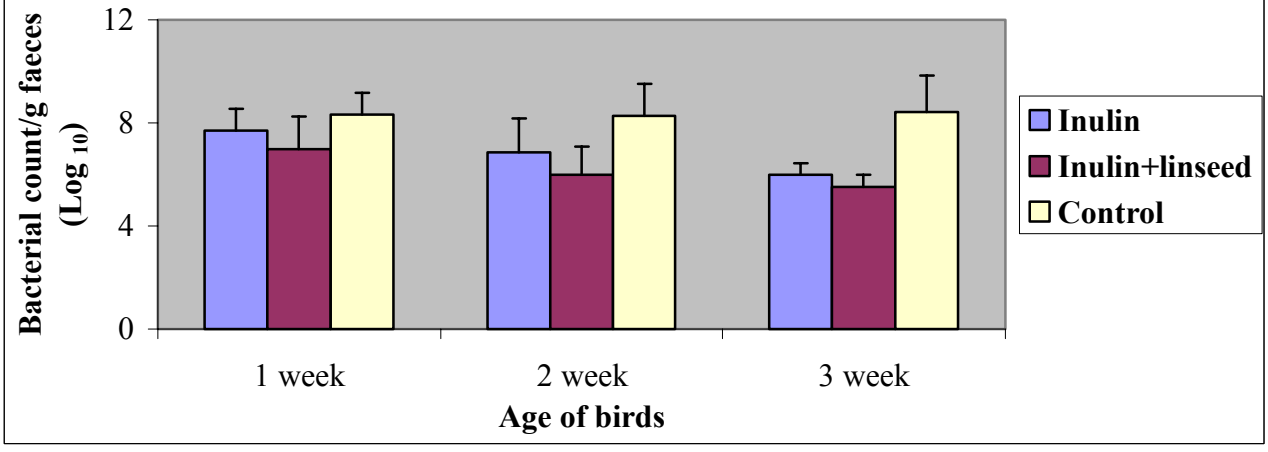


Figure 24. Changes in *Bdellovibrio* counts in the rectum of I-group , IL-group and C-group of SPF chickens

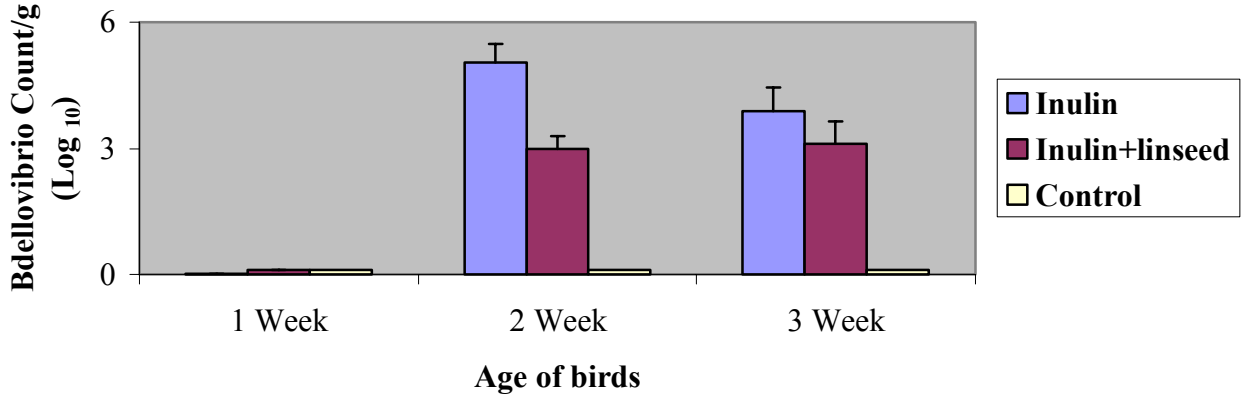


Figure 25. Changes in the body weights (BW) of I-group , IL-group and C-group of SPF chickens

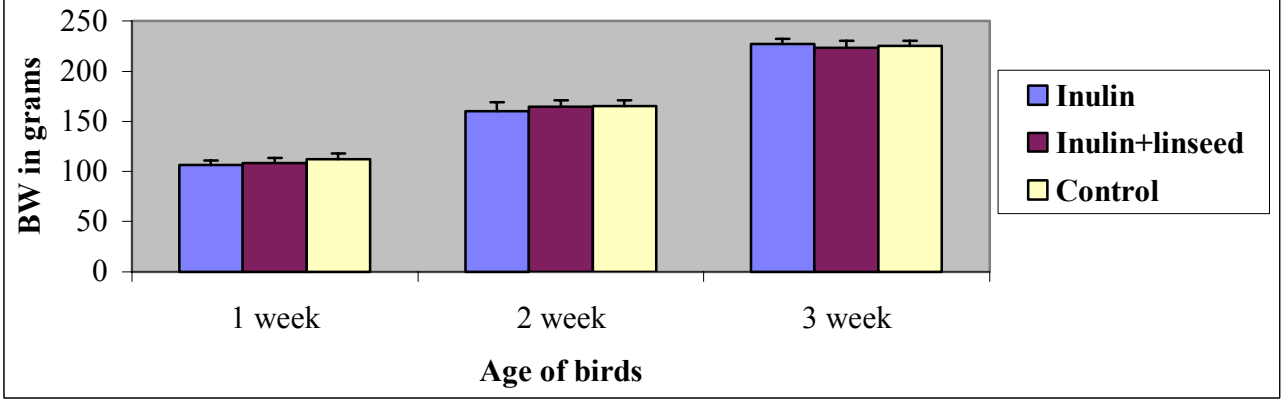
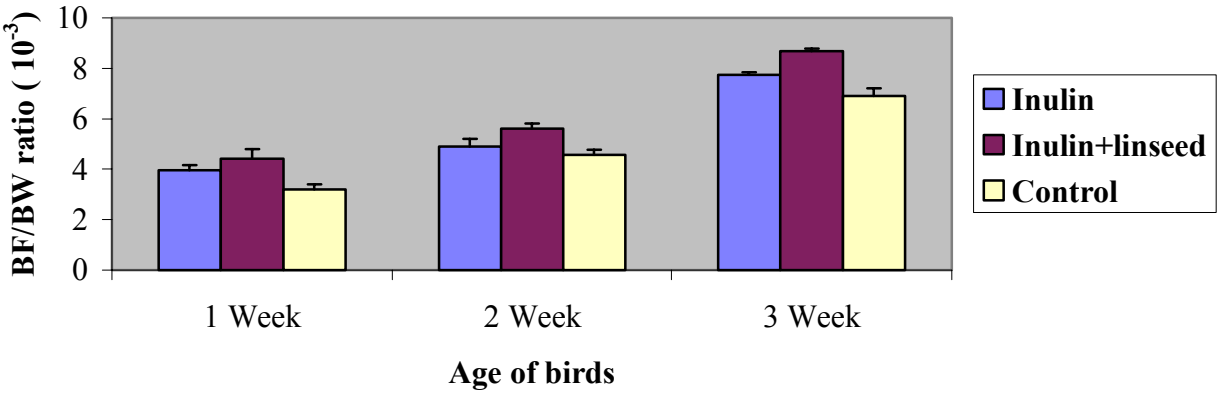


Figure 26. Difference between I-group, IL-group and C-group in BF/BW ratio of SPF chickens

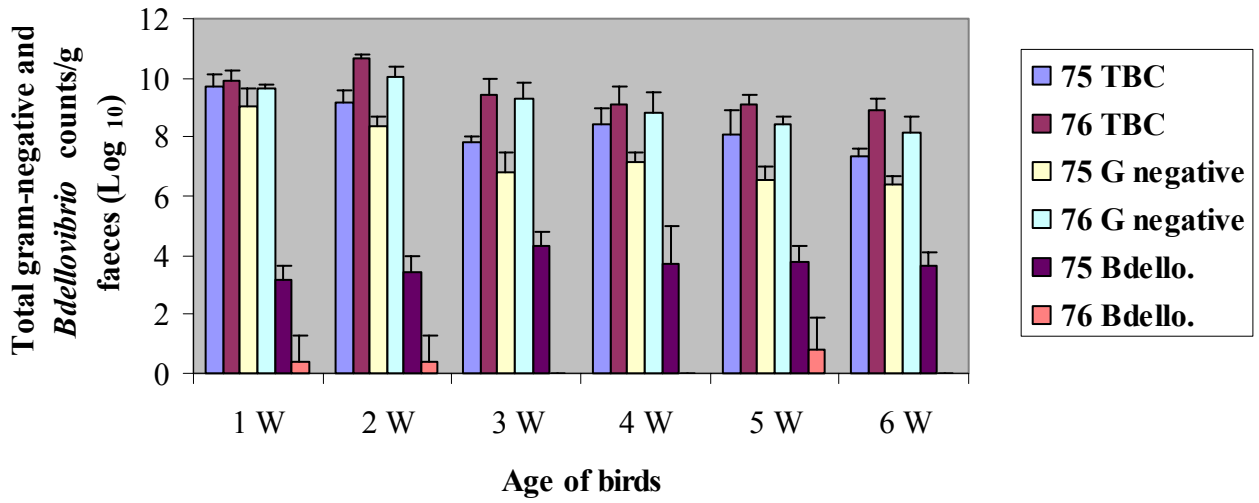


4.3 Effect of inulin (0.5 % via drinking water) on caecal bacterial population, endotoxin and PC-BP serum levels and on the weight BF of broiler chickens (Experiment III)

A) Poultry farm M. I (H. 75 treated and H. 76 control)

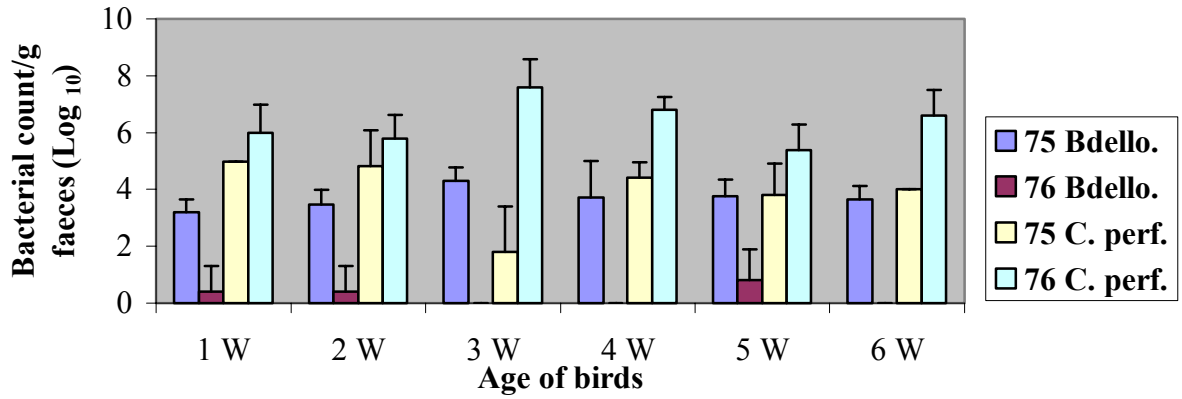
Among the bacterial population enumerated, there was no significant difference in total bacterial count (p=0.28) in the caecum of I-group (with inulin) and C-group (without inulin) after one week of inulin administration but in the second week the total bacterial count by I-group had significantly decreased than C-group (p=0.008). Gram-negative bacterial counts had also significantly decreased after 2 week of inulin administration by I-group (p value ranged from 0.011 – 0.008), whereas the *Bdellovibrio* counts were slightly increased by the I-group (p=0.053) after one week of inulin administration. In the second week and until the end of the experiment (6 weeks) the *Bdellovibrio* counts had significantly increased (p value ranged from 0.010 – 0.005) than C-group, figure (27, 28), table 5A, appendix.

Figure 27. Relation between total (TBC), gram-negative (G negative) and *Bdellovibrio* (Bdello.) bacterial counts in I-group (H.75) and C-group (H. 76) of farm M.



On the other hand, there was a decrease in the *C. perfringens* caecal counts of I-group, which were slightly significant at the first week of the experiment (p=0.05). In the third week there was a very significant decrease in *C. perfringens* caecal counts of the I-group (p=0.006) in comparison with C-group. There was also a negative correlation between *C. perfringens* and *Bdellovibrio* caecal count, figure (28).

Figure 28. Relation between *Bdellovibrio* and *C. perfringens* (perf.) counts in I-group (H. 75) and C-group (H. 76) of farm M.



There were no significant changes in BW between I-group (with inulin) and C-group (without inulin) during the experiment except in the 5th week: there was a significant increase in the BW of I-group (p=0.009), figure (29), whereas there was a significant increase in P/BW and BF / BW ratios (p=0.008) in the I-group after 2 weeks of inulin administration until the end of the experiment, figure (30). The endotoxin level in the blood of I-group was significantly reduced in comparison with C-group especially at the end of the experiment at 6th week (p=0.0356). There was also a significant reduction in PC-BP, p=0.009 in the second week and in the 5th week (p value=0.047) in the blood of the I-group in comparison with C-group, figure (31). There was a negative correlation between PC-BP, endotoxin levels in the blood of birds and growth rate of body weight. Also at increasing total aerobic, gram-negative and *C. perfringens* counts there was an adverse effect on the BW, whereas there was a positive correlation between *Bdellovibrio* count and growth rate of BW of birds.

Figure 29. Difference in the growth of BW between I-group (H. 75) and C-group (H. 76)

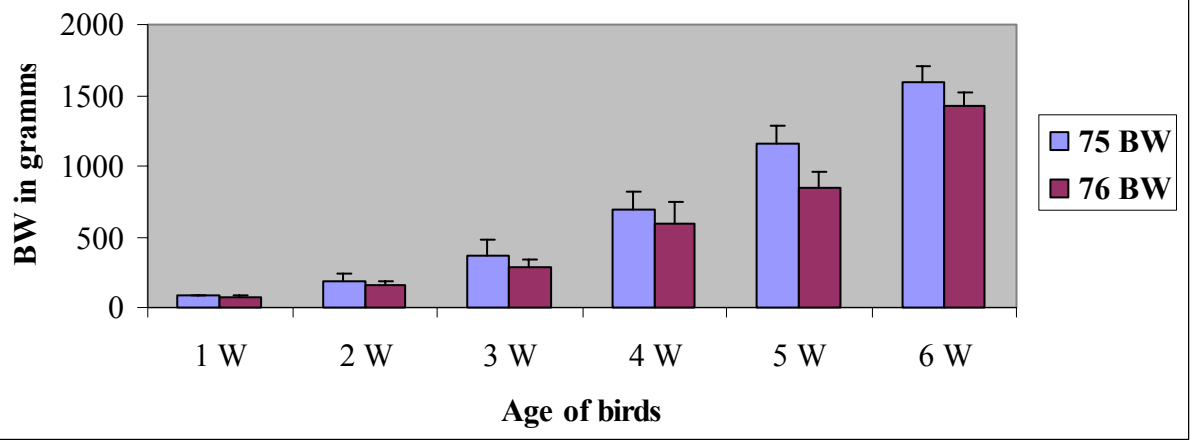


Figure 30. Comparison of P/BW & BF/BW ratios between I-group (H. 75) and C-group (H. 76)

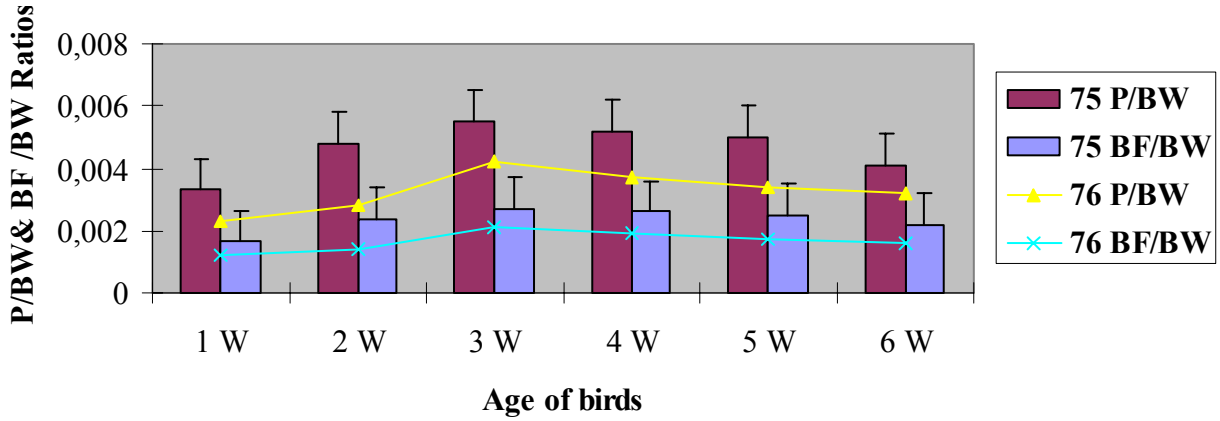
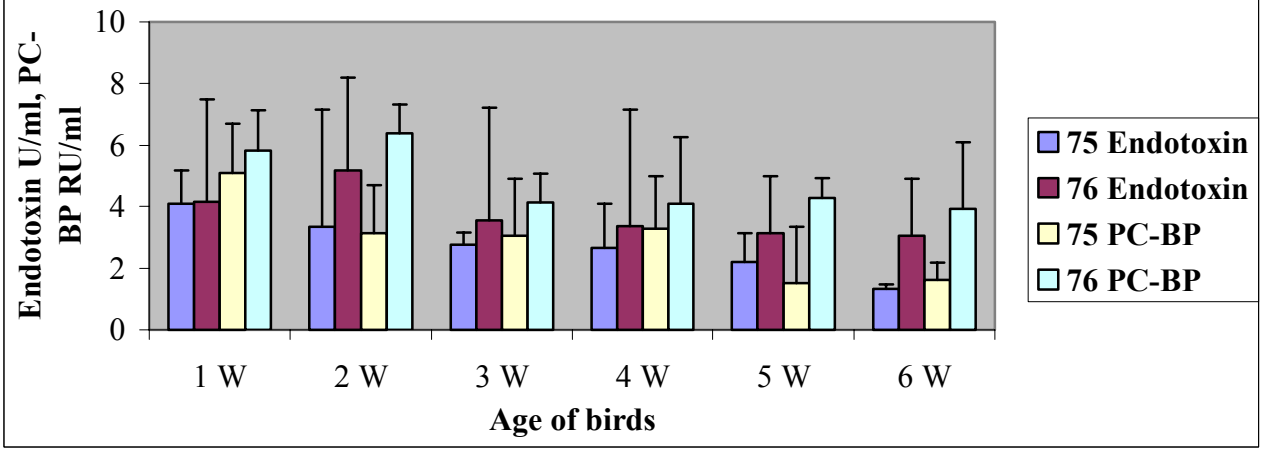


Figure 31. Comparison between I-group (H. 75) and C-group (H. 76) in some blood parameters



B) Poultry farm M. II (Herd 165 and Herd 251) -Experiment III

The bacteriological examinations revealed that; there was no significant difference in the total aerobic bacterial count ($p>0.05$) in the caecum of the I-group and C-group after four weeks of inulin administration but in the 5th week the total aerobic bacterial counts of I-group had significantly decreased than C-group ($p=0.02$). In herd 251 (I-group), there were no significant relation between total and *Bdellovibrio* bacterial counts ($p> 0.05$). In herd 165 (C-group), total bacterial counts were significantly related (positively) to endotoxin and PC-BP blood levels but there was no significant relation between total bacterial counts and gram-negative, *C. perfringens* and *Bdellovibrio* counts.

Gram-negative bacterial counts were also significantly decreased after 2 weeks of inulin administration in I-group (p value ranged from 0.0069-0.009), whereas the *Bdellovibrio* counts were slightly increased by I-group ($P=0.052$) until the second week. From the third week, until the end of experiment (5 weeks) *Bdellovibrio* counts had significantly increased (p value ranged from 0.007 –

0.02) than C-group, figure 32. On the other hand there was a decrease in the *C. perfringens* caecal counts of I-group in the 2nd week of the experiment (p=0.0413) and in the 4th and 5th week there was a strongly significant decrease in *C. perfringens* caecal count of I-group (p=0.0053-0.0062 respectively) in comparison with C-group. There was also a negative correlation between *C. perfringens* and *Bdellovibrio* caecal counts, figure 33, table 7A, appendix.

Figure 32. Relation between total aerobic, gram- negative, and *Bdellovibrio* bacterial counts in I-group (H. 251) and C-group (H. 165)

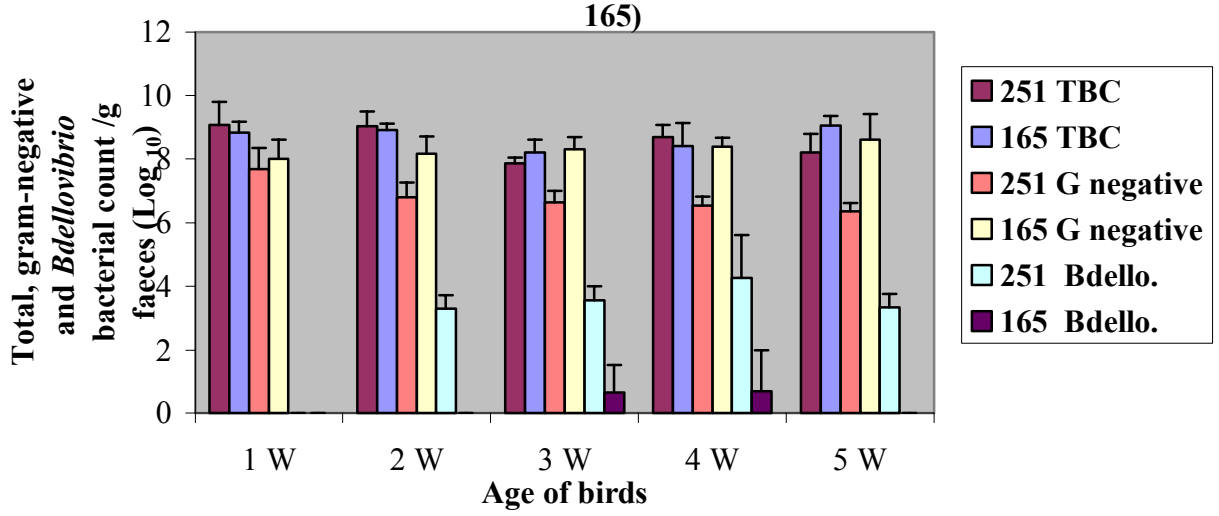
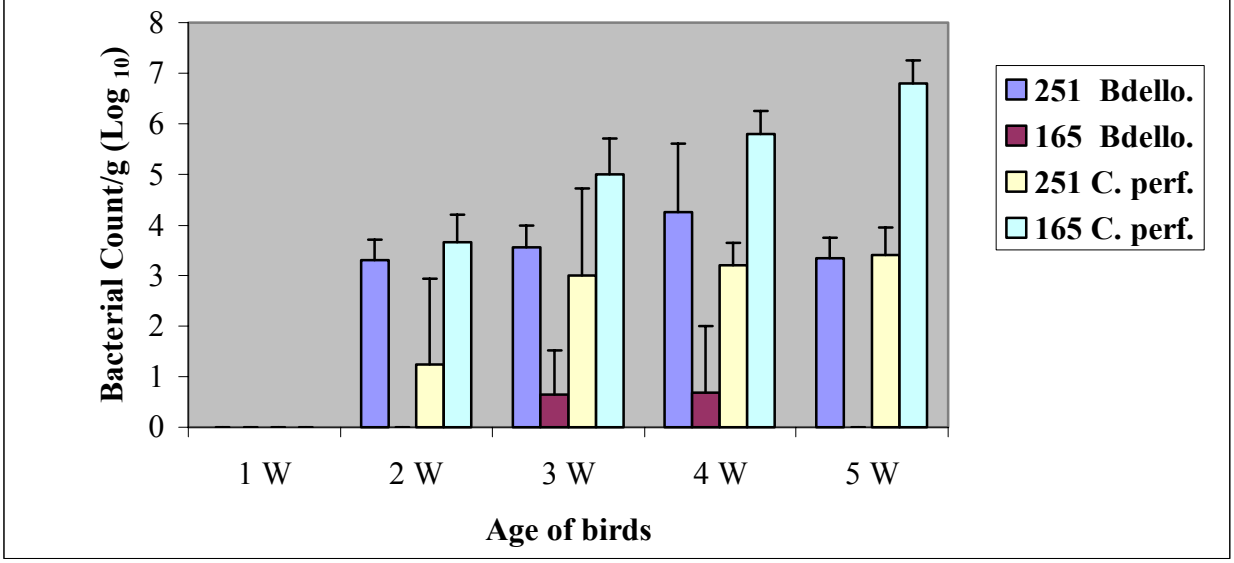


Figure 33. Relation between *Bdellovibrio* and *C. perfringens* counts in I-group (H. 251) and C-group (H. 165)



There were no significant changes in BW between I-group and C-group during the first two weeks. There was a significant increase in BW in I-group (p=0.0160) in the third week, figure 34. There was also a significant increase in P/BW and BF/BW ratios (p=0.0086) in I-group after 3 weeks of inulin administration until the end of the experiment (p=0.0082), figure 35. The endotoxin blood levels in I-group was significantly reduced in comparison with C-group especially at the end of the experiment in the 5th week (p=0.0356). There was also significant reduction in PC-BP blood levels of I-group, p=0.009 in the second, third and the 4th week and by the 5th week p value=0.0086 in comparison with C-group, figure 36. There was a negative correlation between PC-BP, endotoxin blood levels in all groups and the growth rates of BW. Increasing total aereobic, gram-negative and *C. perfringens* counts had adverse effect on the growth rates of BW on, whereas there was positive correlation between *Bdellovibrio* counts and growth rates of BW of birds.

Figure 34. Difference in the growth rates of BW between I-group (H. 251) and C-group (H. 165)

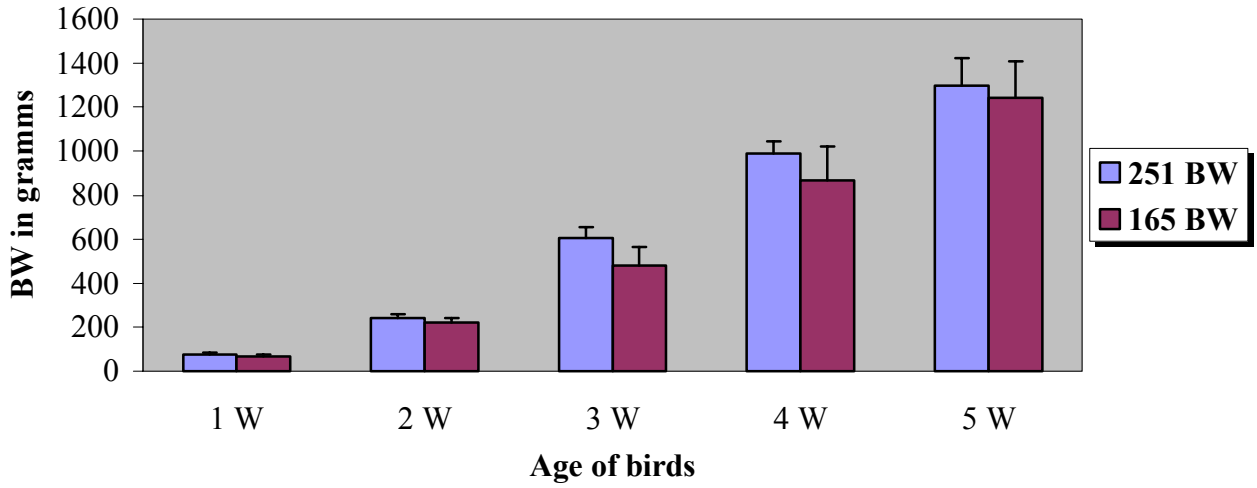


Figure 35. Comparison of P/B and BF/BW ratios between I-group (H. 251) and C-group (H. 165)

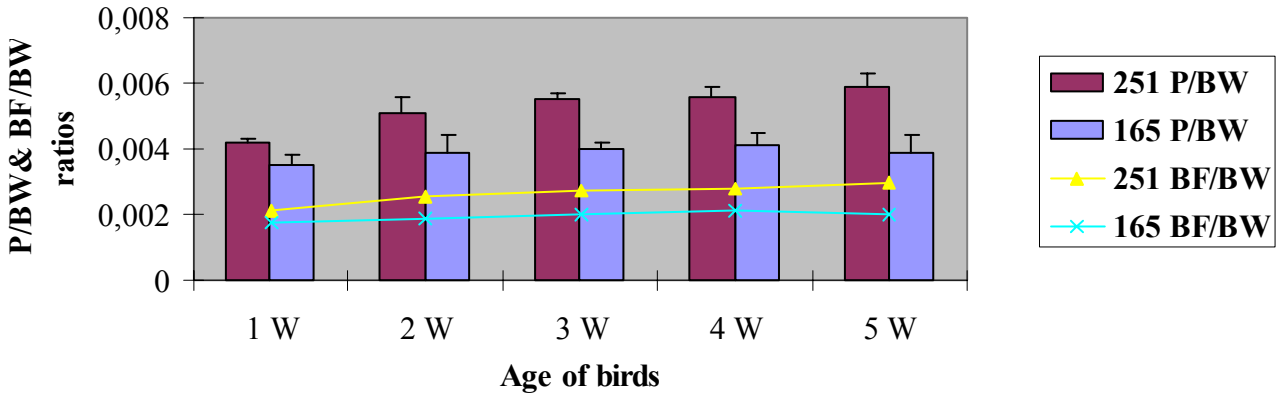
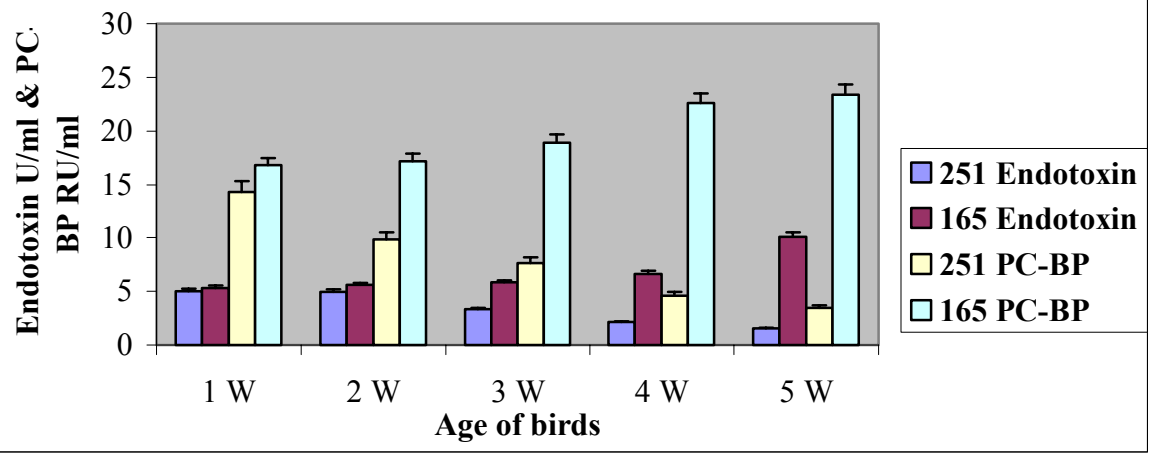


Figure 36. Comparison between I-group (H. 251) and C-group (H. 165) in some blood parameters



4.4 Analysis of some intestinal flora and its relationship to some blood parameters in broilers and breeder chickens (Experiment IV)

4.4.1 In broilers

A) R. I farm

The relation between *C. perfringens* and *Bdellovibrio* caecal counts:

It was found that when the C. *perfringens* ceacal counts were negatively related with the *Bdellovibrio* caecal counts, (Correlation coefficient [r_s] = 0.83, $p<0.01$) figure 37. There was also negative relationship between *Bdellovibrio* caecal counts and endotoxin levels, (r_s = 0.54, $p<0.01$), figure 38. On the other hand *Bdellovibrio* caecal counts were positively related with the P/BW and BF/BW ratios (r_s =0.44, $p<0.02$), whereas the increase in the endotoxin level had a negative effect on these ratios (r_s =0.39, $p<0.05$), figure 39, 40. There were significant relationships between total, gram-negative, C. *perfringens* and *Bdellovibrio* counts with endotoxin but not with PC-BP blood levels, table 7A, appendix.

Figure 37. Relation between *Bdellovibrio* and C. perf. counts in broilers (R. I farm)

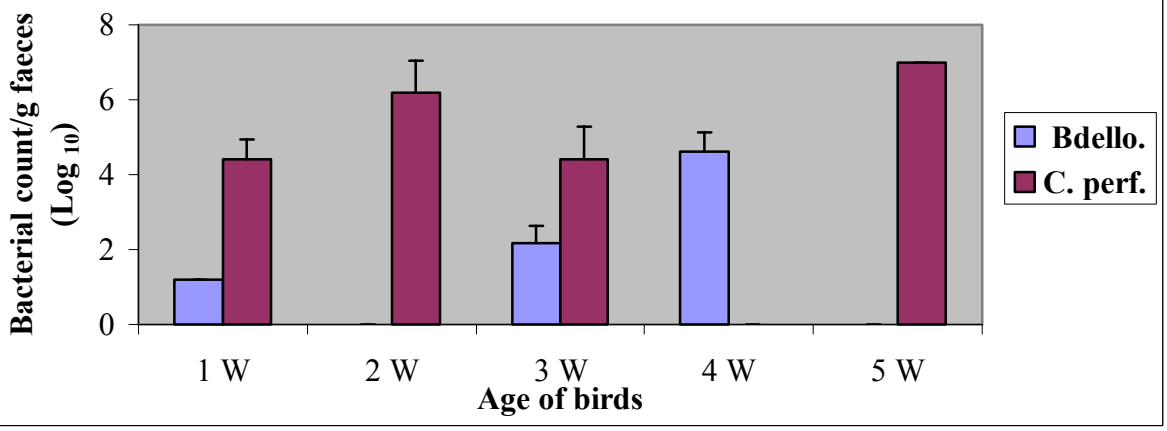


Figure 38. Relation between *Bdellovibrio* caecal counts and endotoxin blood levels in broilers (R. I farm)

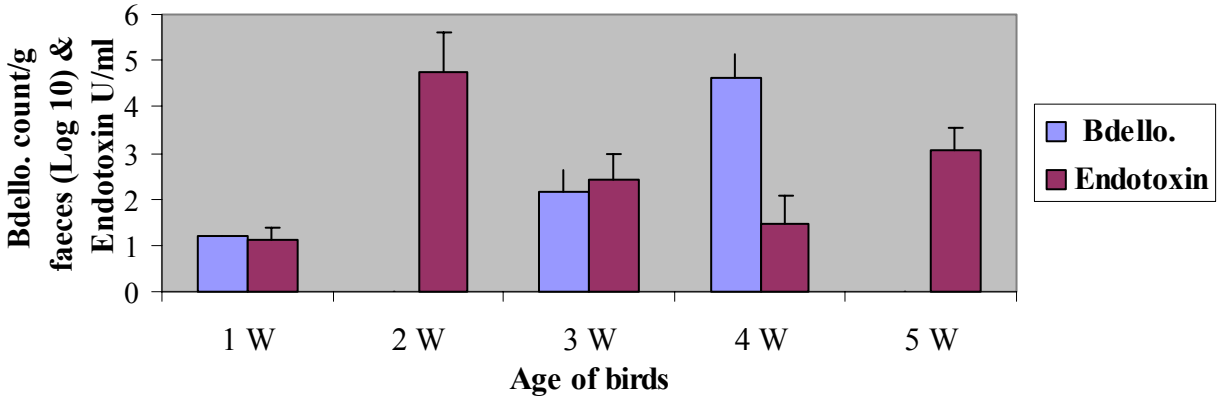


Figure 39. Relation between *Bdellovibrio* caecal bacterial counts and P/BW & BF/BW ratios of broilers (R. I farm)

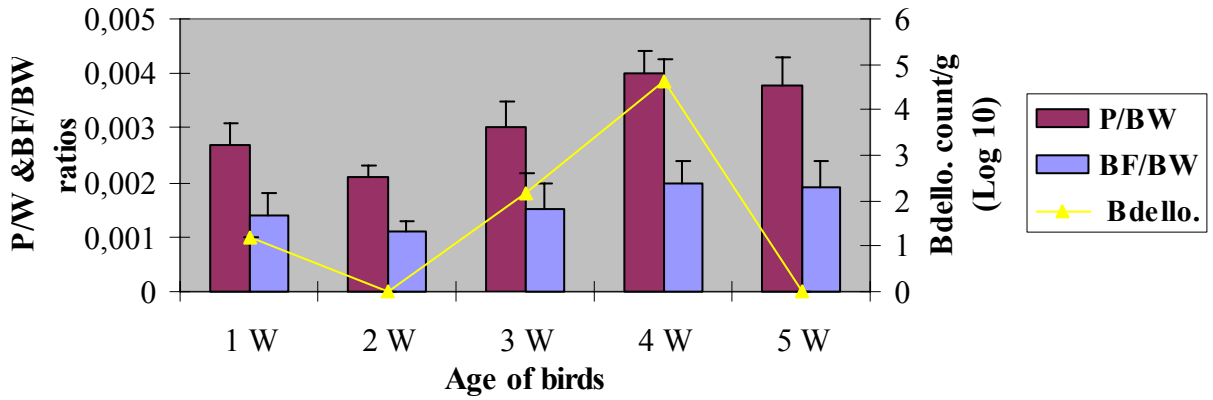
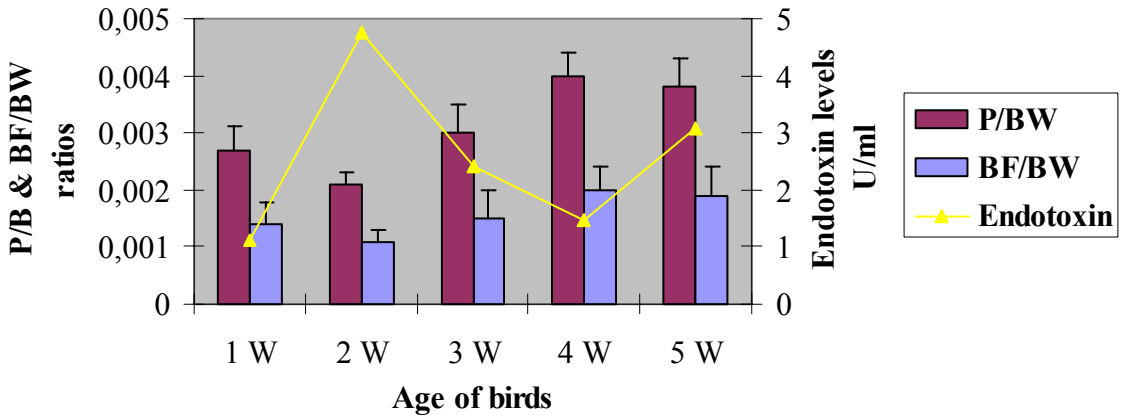


Figure 40. Relation between P/BW & BF/BW ratios and endotoxin blood levels in broilers (R. I farm)



B) R. II farm

It was found, that the *Bdellovibrio* caecal counts were negatively related with the total aerobic, gram-negative and *C. perfringens* counts, (r_s =0.63, 0.66, 0.64, p<0.01, 0.01 and 0.02 respectively) figure 41and 42, whereas the *Bdellovibrio* caecal counts were positively related with the P/BW and BF/BW ratios (r_s =0.70, p<0.01) figure 43, but the endotoxin levels were negatively related with these ratios, figure 44. The total bacterial counts were significantly related to gram-negative, clostridial and *Bdellovibrio* bacterial counts. There was no significant relation between the bacterial parameters (total, gram-negative, *C. perfringens* and *Bdellovibrio* counts) and endotoxin, BC-BP blood levels, table 8A (appendix).

Figure 41. Relation between total aerobic, gram-negative and *Bdellovibrio* bacterial counts in broilers (R. II farm)

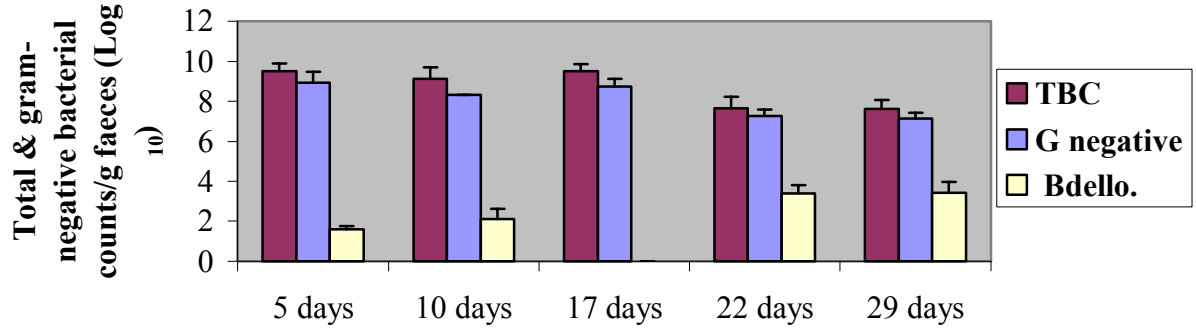


Figure 42. Relation between *Bdellovibrio* and *C. perfringens* counts in broilers (R. II farm)

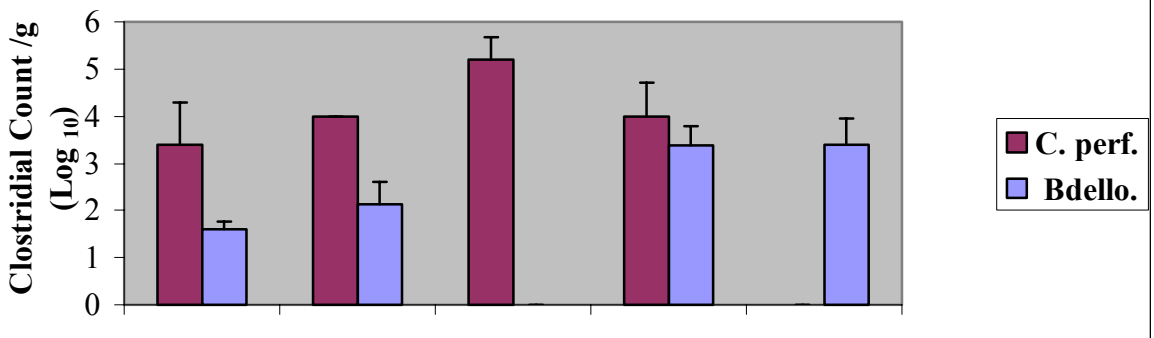


Figure 43. Relation between *Bdellovibrio* bacterial counts and P/BW & BF/BW ratios in R. II farm

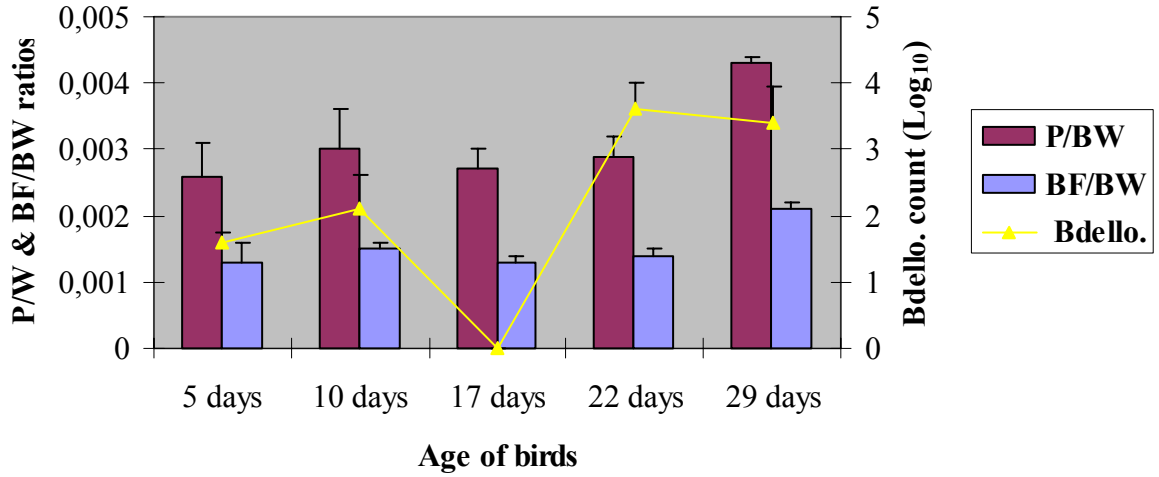
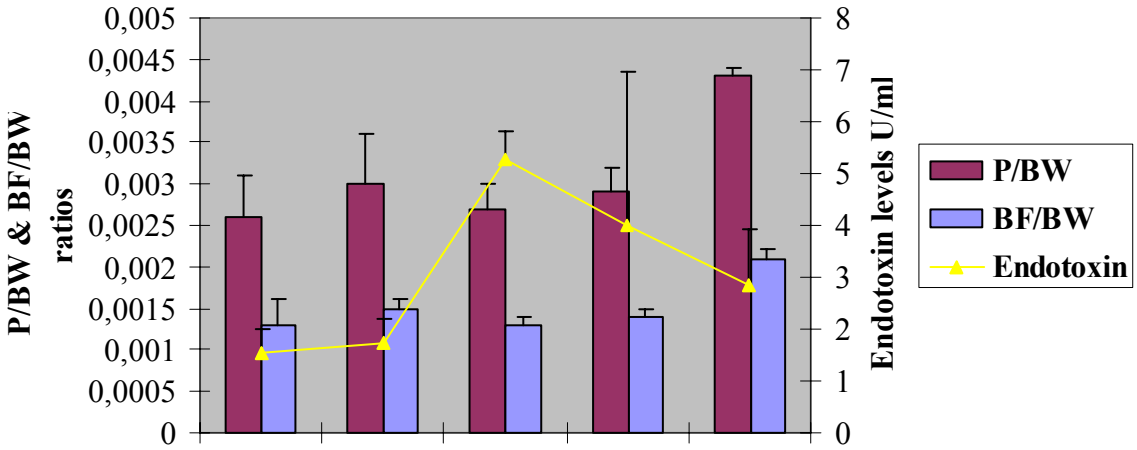


Figure 44. Relation between endotoxin blood levels and P/BW & BF/BW ratios in (R. II farm)



C) Ro. farm

It was found that the *Bdellovibrio* caecal counts were negatively related with the total aerobic, gram-negative and clostridial bacterial counts (r_s =0.52, 0.55, 0.47, $p<0.01$, 0.01 and 0.05 respectively) figure 45 and 46. P/BW ratios were positively related with BF/BW ratios (r_s =0.83, $p<0.01$) figure 47. The endotoxin blood levels were positively related to PC-BP level, figure 48. The total, gram-negative, C. *perfringens*, and *Bdellovibrio* counts were not significantly related with P/BW and BF/BW ratios but significantly (negative correlation) related with endotoxin and PC-BP blood levels (r_s =0.82, $p<0.05$), table 9A, appendix.

Figure 45. Relation between total aerobic, gram-negative and *Bdellovibrio* bacterial counts in broilers (Ro. farm)

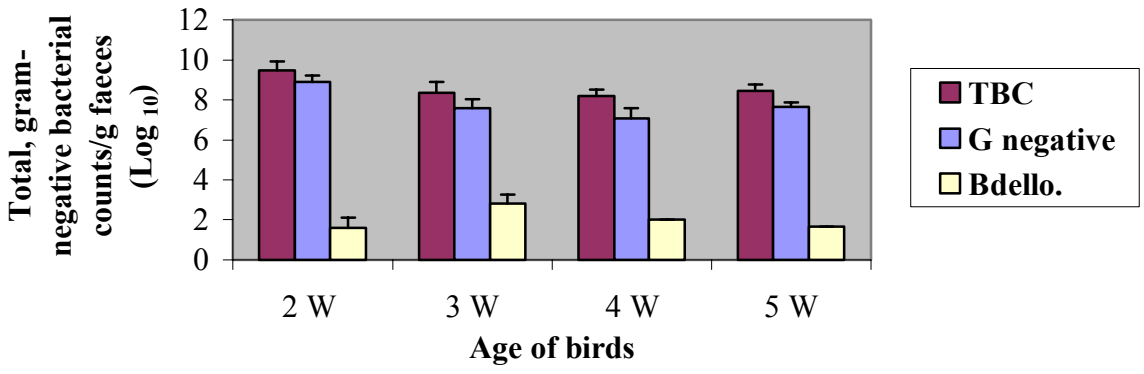


Figure 46. Relation between *Bdellovibrio* and *C. perfringens* counts in broilers (Ro. farm)

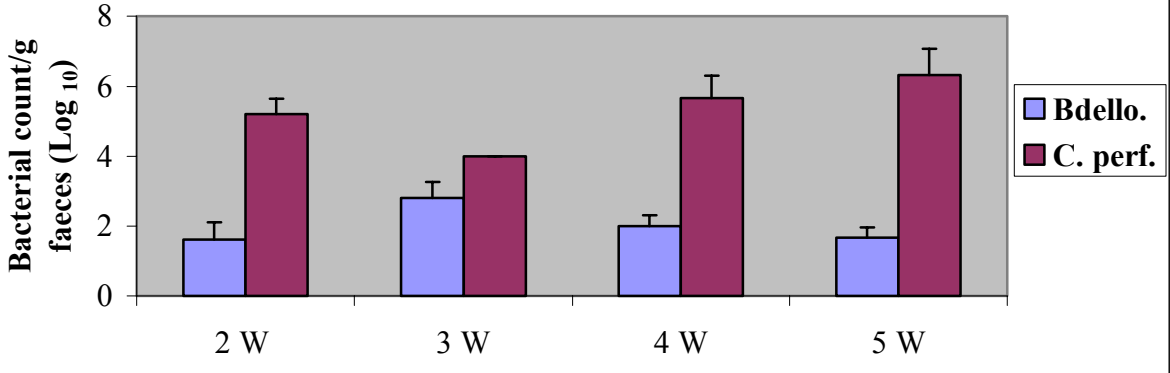
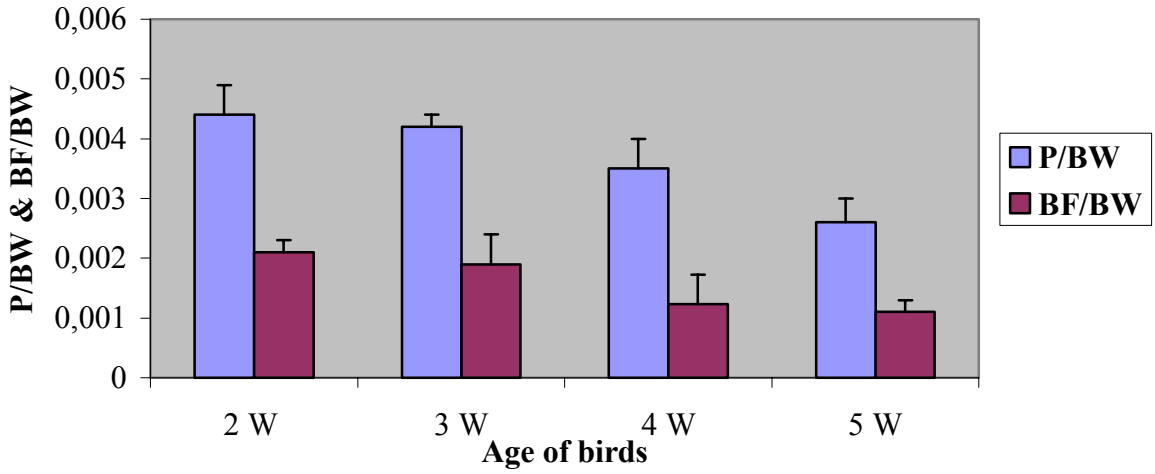
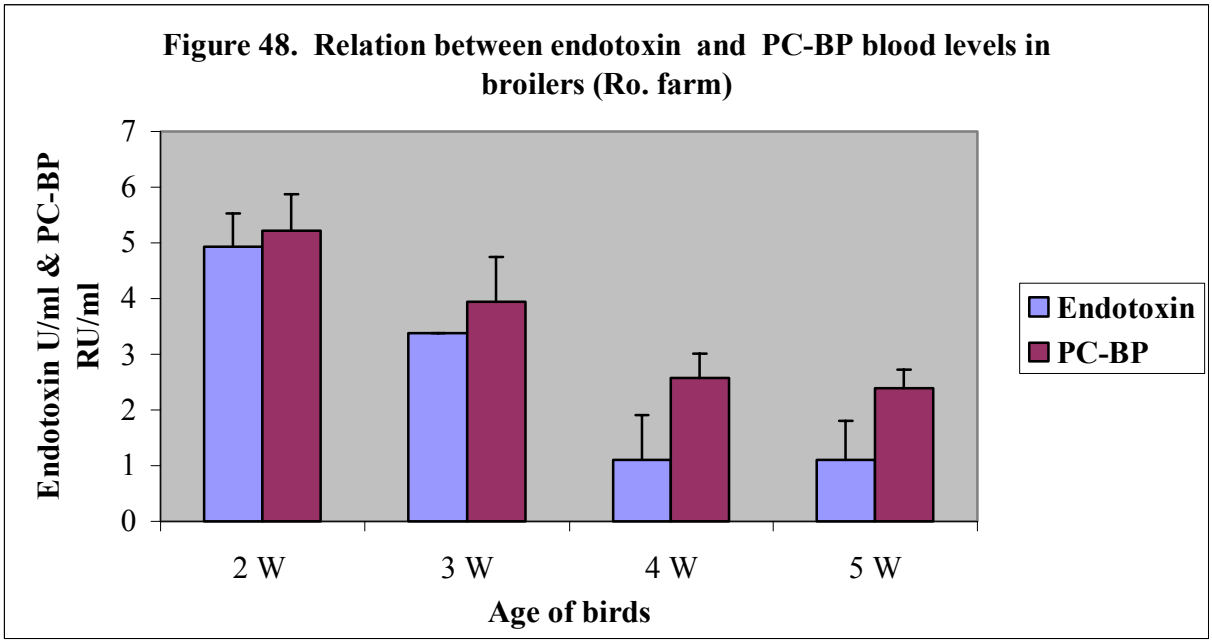


Figure 47. Relation between P/BW and BF/BW ratios in (Ro. farm)





D) Co. farm

It was found that the *Bdellovibrio* caecal counts were negatively related with the *C. perfringens* counts, figure 49. P/BW ratio was positively related with BF/BW ratio (r_s =0.83, $p<0.01$) figure 50. The endotoxin blood levels were also found positively related to the increasing in total aerobic and gram-negative bacterial counts and PC-BP levels (r_s =0.55, 0.48, 0.70, $p<0.01$, 0.02 and 0.01 respectively) figure 51 and 52. The total, gram-negative, *C. perfringens* counts were not significantly related with P/BW and BF/BW ratios ($p> 0.05$) but significantly related with endotoxin blood levels (r_s =0.61, $p<0.05$), table 10A, appendix.

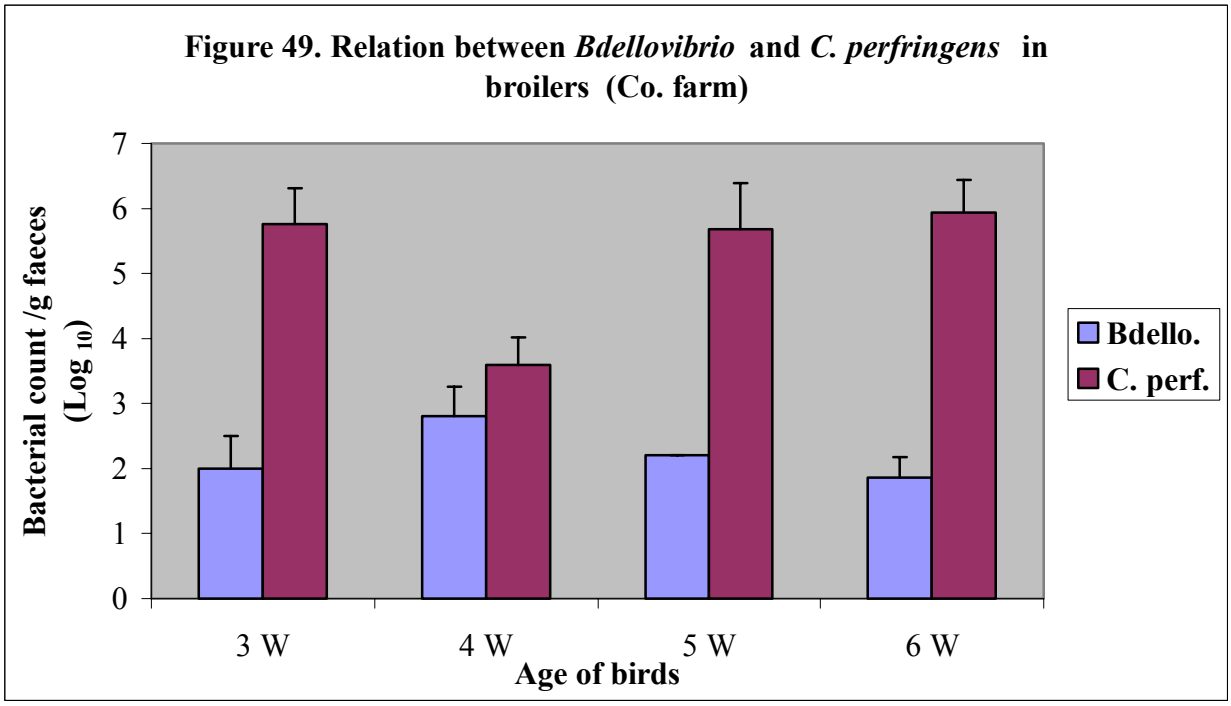


Figure 50. Relation between P/BW & BF/BW ratios in broilers (Co. farm)

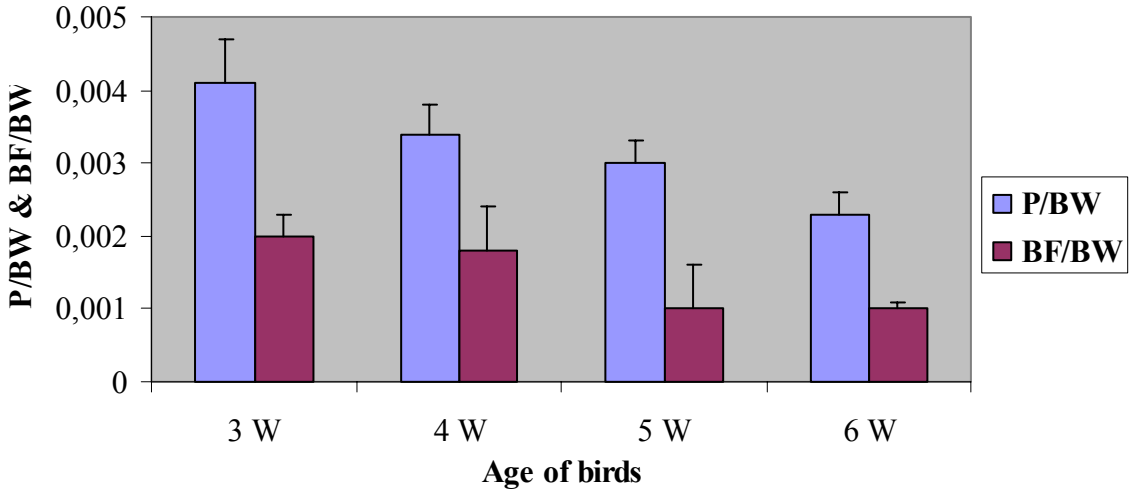


Figure 51. Relation between total aerobic, gram-negative bacterial counts and endotoxin levels in broilers (Co. farm)

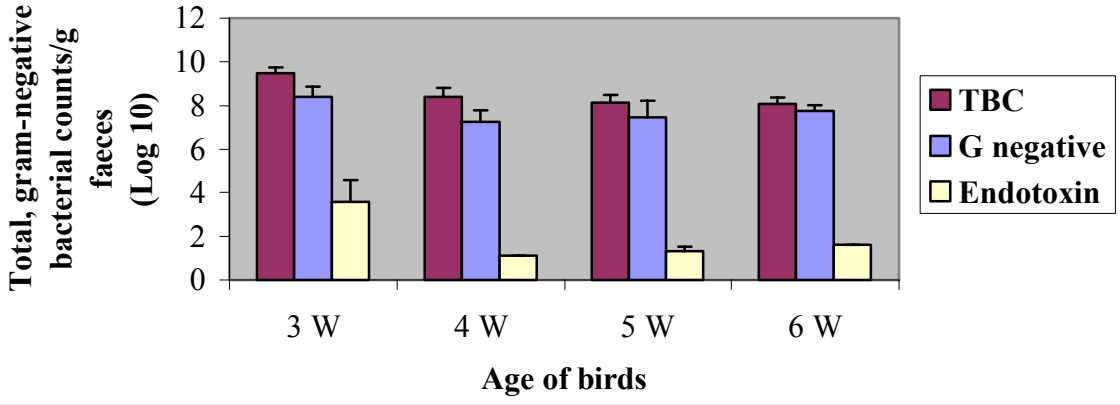
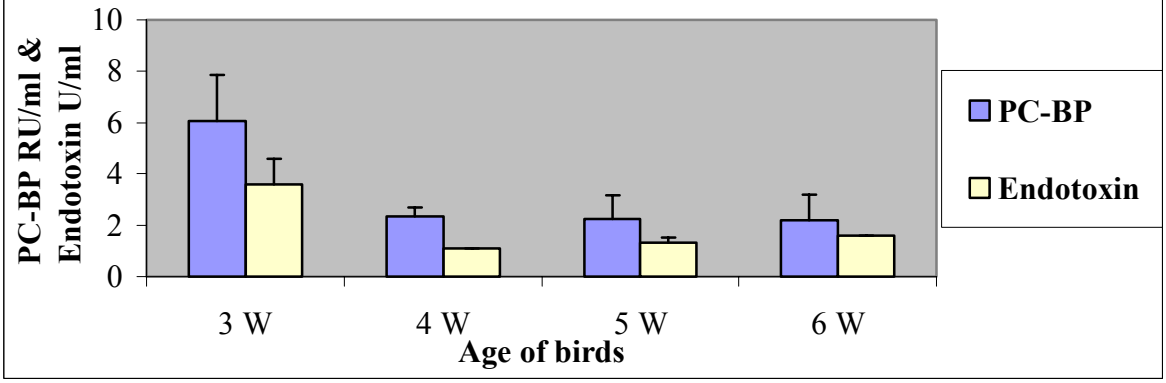


Figure 52. Relation between PC-BP and endotoxin blood levels in broilers (Co. farm)



4.4.2 In breeders

A) Ho. farm

It was found that the *Bdellovibrio* caecal counts were negatively related with the clostridial bacterial counts, r_s =0.59, p<0.01, figure 53. *Bdellovibrio* caecal counts were also positively related with the BW, r_s =0.53, p<0.002, figure 54. The PC-BP levels were also positively related with the endotoxin levels, r_s =0.37, p<0.050 and with an increase of caecal clostridial count, r_s =0.55, p<0.002, figure 55 and 56. The total, gram-negative and *Bdellovibrio* bacterial counts were not significantly related with P/BW and BF/BW ratios (p> 0.05) and also with the endotoxin and PC-BP blood levels (p<0.05), table 11A, appendix.

Figure 53. Relation between caecal *Bdellovibrio* and *C. perfringens* counts in breeders (Ho. farm)

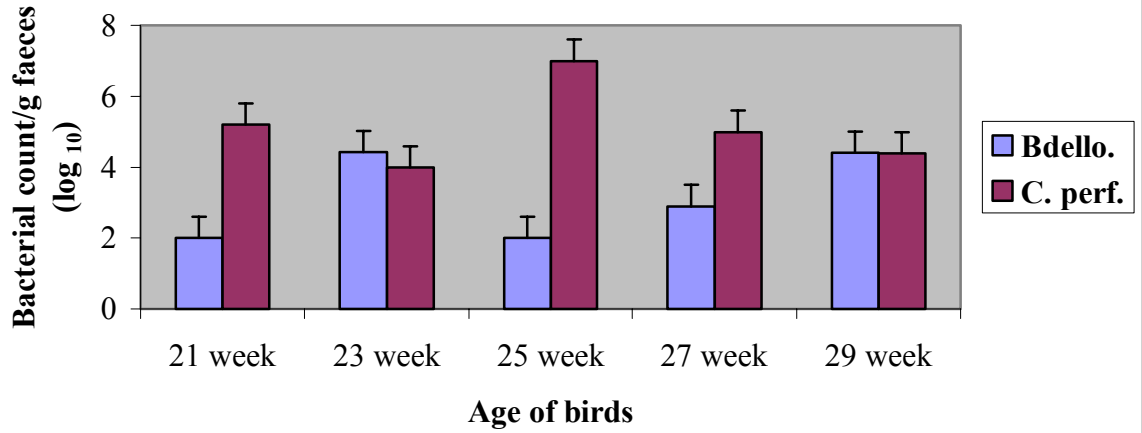
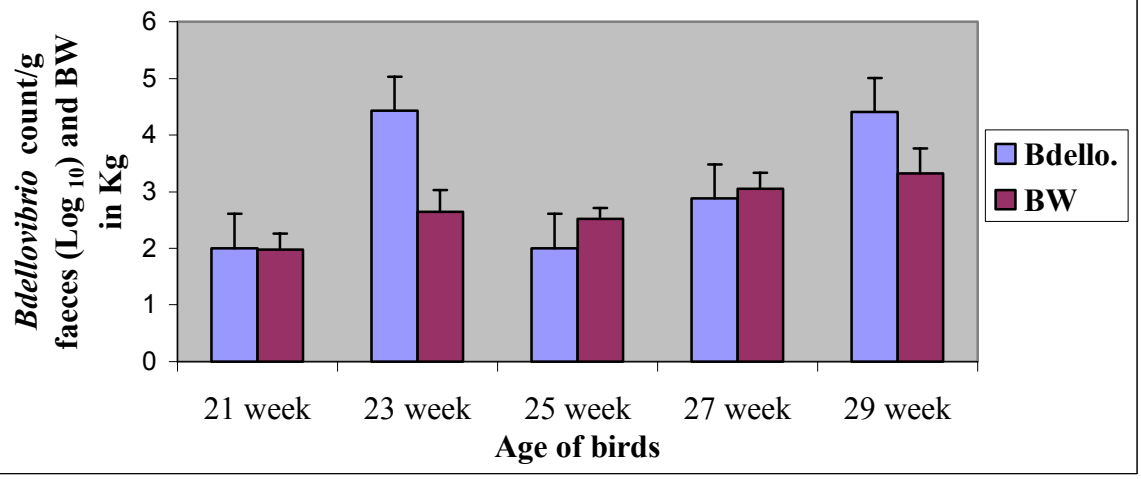
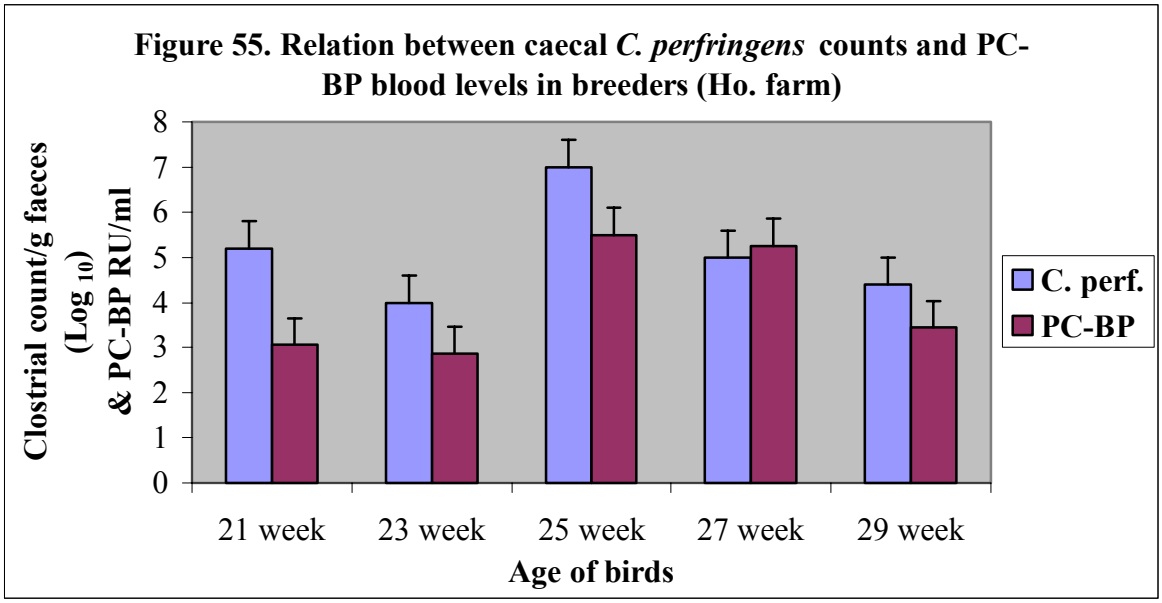
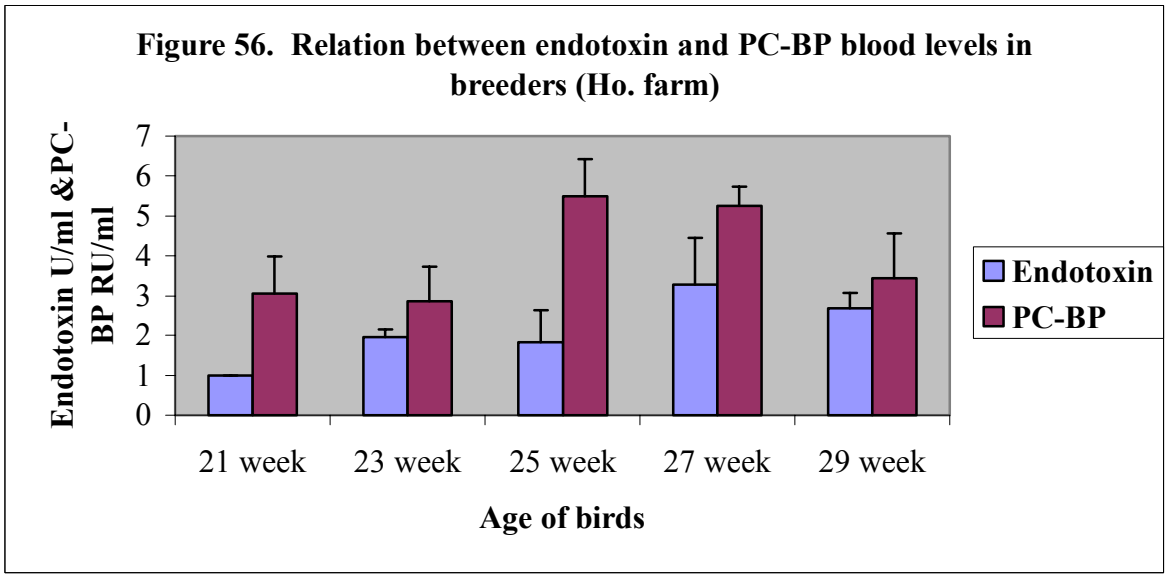


Figure 54. Relation between caecal *Bdellovibrio* counts and BW of breeders (Ho. farm)







B) Wa. farm

It was also found, that the caecal *Bdellovibrio* counts were negatively related with the total aerobic, gram-negative and the *C. perfringens* counts, figure 57 and 58. The PC-BP levels were also positively related with the endotoxin levels, r_s =0.52, $p<0.007$ and with increasing of caecal *C. perfringens* counts, r_s =0.48, $p<0.014$, figure 59 and 60. The total, gram-negative and *Bdellovibrio* bacterial counts were not significantly related with P/BW and BF/BW ratios and with the endotoxin and PC-BP blood levels ($p> 0.05$), table 12 A appendix.

Figure 57. Relation between total aerobic bacterial, gram-negative and *Bdellovibrio* bacterial counts in breeders (Wa. farm)

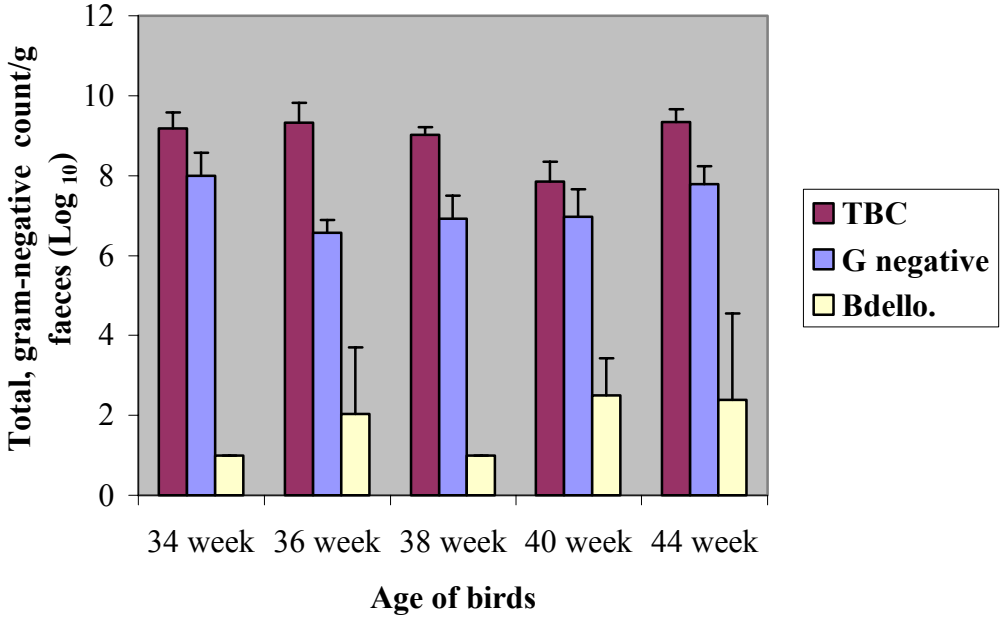


Figure 58. Relation between caecal *Bdellovibrio* and *C. perfringens* in breeders (Wa. farm)

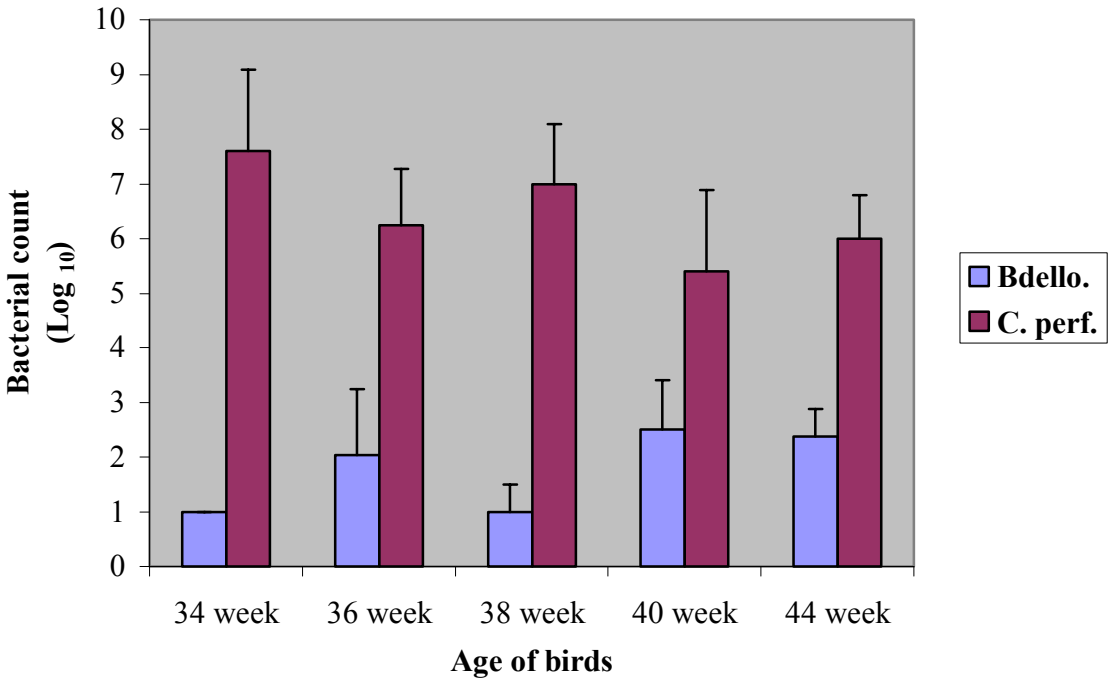


Figure 59. Relation between caecal *C. perfringens* counts and PC-BP blood levels in breeders (Wa. farm)

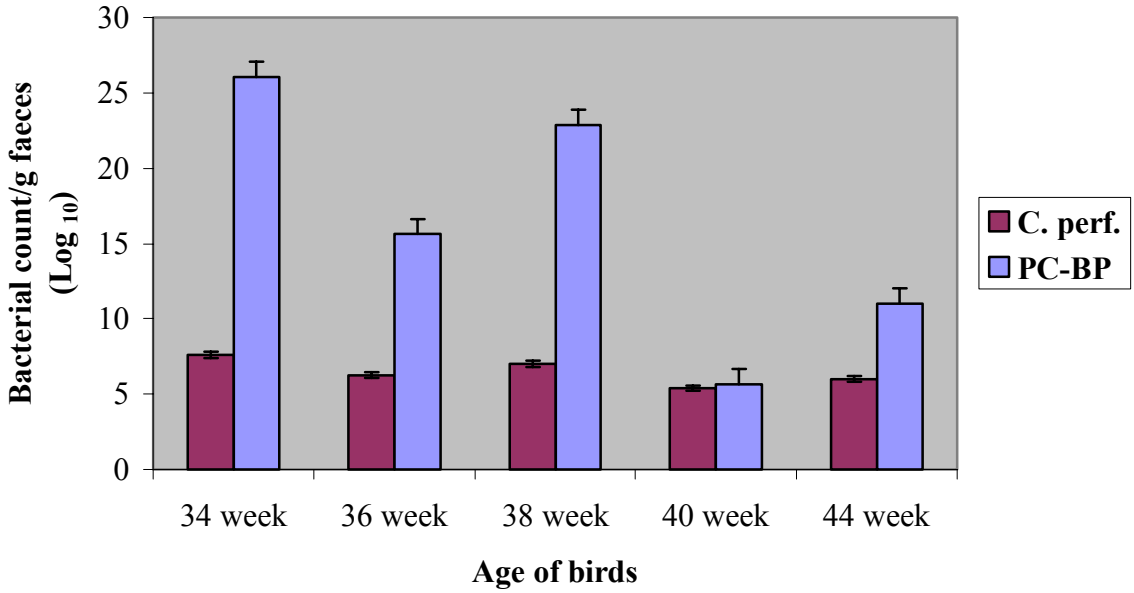
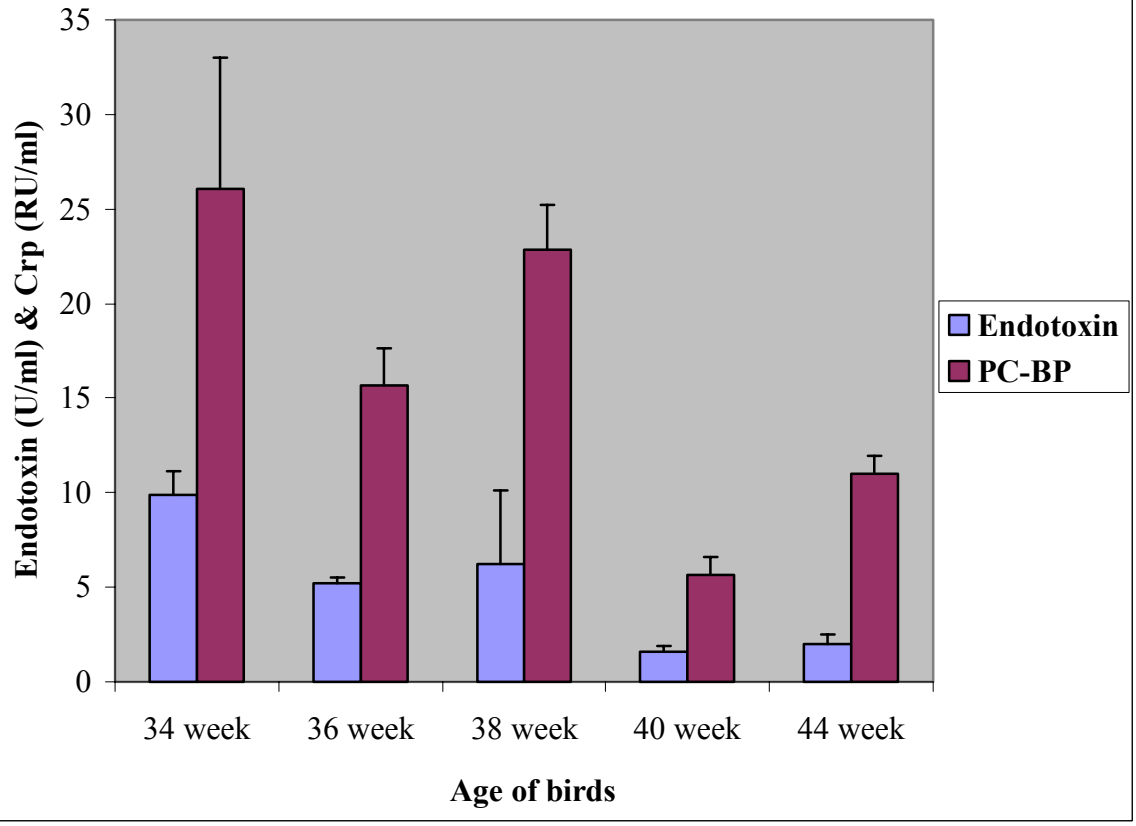


Figure 60. Relation between endotoxin and PC-BP levels in the blood of breeders (Wa. farm)



5. Discussion

5.1 Critical discussion of used methods

5.1.1 Selection of the suitable part of the digestive tract for bacteriological investigations

Our results showed that the caecal contents in each age of chicken are the suitable material for bacteriological investigations. We have found significant differences between control and trial groups in total bacterial and gram-negative bacterial counts at the third week and in *Bdellovibrio* counts from the second week until the end of the experiments. From the microbiologist's standpoint, the intestine can be divided into three sections:

The duodenum, where the numbers of bacteria are relatively low, generally less than 10^8 /g intestinal content.

The caeca, where a considerable microbial fermentation occurs, the number of bacteria present being approximately 10^{11}/g caecal contents.

The large intestine, which in most birds is relatively short and includes organisms from both the small intestine and caeca.

In all three parts of tracts, microbial habitats may exist in any area from the oesophagus to the cloacae. Some of these habitats may cover visible areas of the mucosal epithelium; others may be of microscopic dimension, i.e. microhabitats. They may occur in any major area of the tract, in the lumen, on an epithelial surface, or deep in the crypts of Lieberkuhn in the mucosa. The lumen can be colonized by microbes in any area of the tract but may be colonized normally only in areas of relative stasis, such as the caecum, and large intestine, where the now rate of the contents does not exceed the doubling rate of the microbial population levels (SAVAGE 1977). Therefore we have selected all parts of the digestive tract for the early studies in the SPF chickens and for the successive studies on the broilers or breeder chickens we have selected only the caecum. The caecal contents of chicken present homogenous substrate and that also in sufficient amounts for quantitative examination in all groups of age.

5.1.2 Selection of the bacteriological parameters

Prior to hatching, the intestinal tract of the chicks is usually sterile and the intestinal flora is derived exclusively from the environment. Within a few hours of hatching, Enterococci, Enterobacteria and sometimes clostridia, can be found multiplying in the caeca and scattered randomly throughout the rest of the alimentary tract. With coprophagic birds, such as the domestic fowl, the transfer of bacteria from parent to chicks occurs very efficiently and allows the young animal to establish a protective intestinal flora within the first couple of days after hatching (SMITH 1965).

High levels of faecal enterococci and enterobacteria persist for several days in the duodenum, the lactobacilli become established only by about the third day, but by the seventh day they have almost completely replaced the other bacteria. Faecal enterococci often found with the lactobacilli in the small intestine but very high numbers of coli-bacteria or clostridia are unusual. The caecal population of the 1-day-old chicks is predominated by enterococci and coliform bacteria. Onwards

the initial flora was shown to consist almost entirely of faecal enterococci and coli-bacteria with lactobacilli slowly increasing in numbers. After about 14 days of age all three groups are gradually replaced by an anaerobic flora, their numbers falling to below 10^9 /g intestinal content. The caecal flora continues to change for several weeks and shows increasing in the complexity (SMITH 1965 BARNES et al. 1979, SPRING 1997). At this time bifidobacteria, bacteriodes, eubacteria, peptostreptococci and clostridia predominate in the caeca (BARNES et al. 1979). The proof methods of the total aerobic, gram-negative and *C. perfringens* counts (as species of the anaerobes) do not need much technical effort and significant to evaluate the changes in the caecal populations. Bdellovibrios, because of their lytic properties, play an important role in maintenance of homeostasis in the ecological system. This proves the participation of bdellovibrios in metabolic cycle of nature. Under application of antibiotics and under presence of inflammation cascades is the gastrointestinal *Bdellovibrio* flora disturbed, and this leads to bacterial overgrowth (dysbiosis) in particular in caecum area and to translocation of bacteria and bacterial particles (LPS, peptidoglycan) (EDAO et al. 1998). Therefore we have selected this parameter (*Bdellovibrio* bacterial count), to study the effect of prebiotic (inulin) on the changes of *Bdellovibrio* bacterial counts and the role of the *Bdellovibrio* in the ecosystem.

5.1.3 Selection of blood parameters

5.1.3.1 Bacterial endotoxins lipopolysaccharide

The endotoxin macromolecules are cell wall's components of the gram-negative bacteria. The endotoxins are produced by gram-negative bacteria of intestinal flora. If the endotoxins are translocated from the intestinal tract to the circulation or injected into blood stream, they elicit (depending from the quantity of endotoxin), slight or serious effects (e.g. endotoxin shock). In the effects of endotoxin certain cell populations (e.g. thrombocytes, macrophages, leukocytes, etc.), certain organs and organ-systems (e.g. liver, spleen, pancreas, bone marrow, endocrine and lymphoreticular systems etc.) are involved. Effects of endotoxin are produced by mediators (e.g. endotoxin binding proteins, cytokines, prostaglandins, prostacyclins). The endotoxin sensitivity of vertebrate organisms is dependent from the phylogenetical status of the species. Most sensitive species are the humans and horses. Generally accepted that endotoxin has an important role in the pathogenesis of septic shock. In other pathological processes (e.g. intestinal syndrome of radiation disease, gram-negative infections, various shock forms etc.) are supposed or proved the role of endotoxins. LAL methods are good tools for demonstration the role of endotoxin in the pathogenesis of various processes. For this reason, the experimental endotoxin shock is used a model of septic and other shocks (BERTOK 1998). After endotoxin challenge, the level of enteroadherent bacteria was increased and bacterial translocation was observed (KATAYAMA et al. 1997).

Endotoxins are cell-associated substances that are structural components of the outer membrane of gram-negative bacteria. However, endotoxins may be released from growing bacterial cells or from

cells, which are lysed as a result of effective host defense (e.g. BPI) or the activities of certain antibiotics (e.g. penicillins and cephalosporins) (ALEXANDER and RIETSCHEL 2001).

5.1.3.2 Phosphoryl Choline-Binding Protein (PC-BP)

The PC-BP corresponds in his effect the C-reactive protein (CRP) or acute phase proteins (APP) of mammals. ELFLEIN et al. (1998) had doing preliminary investigations using CRP to assess diseases in poultry. He developed a detection system for quantitative measurement of chicken C-reactive protein CRP from body fluids of healthy and ill animals of varying age. For the first time the CRP has been determined quantitatively in serum and plasma of chicken, turkey, hen, and pigeon as well as in preparations of hatched eggs. For this purpose a species specific ELISA has been developed for chicken-CRP. The detection range extends from 12 to 9,000 µg /l. The lower detection limit is 5 µg /ml. The standard deviations of dilution steps of calibrate curve are 15 per cent on average. The often most intensive lipenic serums and plasmas of chicken showed no significant influence of CRP measurement by using that test system. The calibration of the ELISA has been realised by prepared chicken-CRP. By means of this CRP-ELISA there was a system that detects the status of the unspecific immunsystem influencing stress factors of chicken.
SCHRÖDL et al. (1997) pointed the suitability of the use of PC-BP as alternative parameter for CRP measurement (SCHRÖDL et al. 1997).

Bacterial or parasitic infections, physical and chemical traumata, immunological disorders and malignant tumour growth lead to a highly complex reaction of the organism, called acute-phase response. During acute phase response plasma APP undergo to quantitative changes. Analyses of APP levels and their profile of glycosylation have proven to be very useful in diagnosis, differentiation and monitoring of treatment of a number of human diseases. The levels of CRP may increase in both acute and chronic inflammation (JOHNSON et al. 1999).

The acute phase response is a non-specific, early phenomenon, in which the concentration of a number of plasma proteins is increased following most forms of tissue injury, bacterial infection, inflammation, malignant neoplasia and surgical procedures. These plasma proteins are collectively termed acute-phase proteins. Their most important role is to restore the homeostasis of human organism. CRP regarding his dynamic reaction is one of the best non-specific biochemical indicators of most disorders. The multiple quantitative measurement of CRP is useful in the diagnosis, differentiation, the assessment of treatment effectiveness and patient's prognosis, additionally to other clinical parameters (SZCZEPANEK et al. 1997).

It has been suggested that determining the concentrations of acute phase proteins could help to monitor poultry health. Production-associated subclinical diseases are difficult to detect by post-mortem analysis but might be diagnosed by the presence of changed levels of APPs. In animals the acute phase response is linked to growth depression and decreased production.

In general, avian species react comparable to mammals on inflammation, infectious diseases or other changes of the homeostasis, although the reaction of APP in birds is different from the reaction in mammals (TOUSSAINT et al. 2001).

5.1.4 Selection of P/BW and BF/BW Ratios as immunological parameters

The development of body weight is a fundamental character of animal health. The homeostasis of the GIT flora has essential influence to the development of the body weight. The relationship between the body weight and the weight of primary lymphoid organs had been shown in our investigations. The use of P/BW and BF/BW ratios are a suitable parameter for a statement of the immune status of birds. The pancreas has diffuse lymphoid cells scattered in the parenchyma (SHARMA 1997). Pancreas produces hormones, which controls the balance of glucose between the blood and the rest of the body. This balance keeps the body healthy. The pancreas also produces digestive juices, which helps digest food, so that the body can utilise it. The pancreas has two main roles in the body: It produces juices that contain chemicals (enzymes). These enzymes are lipases: digest fats, proteases**:** digest proteins and amylases digest starches and sugars. These enzymes are collected into the pancreatic duct and are released into the intestine through the duct opening. Once in the intestine, the enzymes help to digest food. It also produces insulin, a hormone that helps to control the level of glucose sugar in blood. Disturbance of this organ e.g. because of bacterial endotoxins may causes disturbance in the body weight and health conditions of human and animals. The human digestive tract contains as much lymphoid tissue as the spleen and as many as 80 of the immunoglobulin-producing cells in the human body are in the intestinal mucosa (BRANDTZAEG et al. 1989). Such a figure is not available for birds, but it is likely to have a close estimate. Lymphoid tissue is scattered through the upper digestive mucosa, at the proventriculus-gizzard junction, and in the duodenum, distal ileum, and caecal tonsils (CLICK 1986). The lamina propria of the gastrointestinal mucosa is rich in lymphocytes. Birds differ from mammals in that there is not a continuing supply of B cells following bursal involution. This discontinuance of supply has implications for B cell renewal from postbursal B cells (WEBER and EWERT 1986) that would seemingly implicate a role for B cells resident in the digestive tract. An intact immune response is an important deterrent to production-limiting protozoan, bacterial and viral diseases.

In poultry, digestive immunosuppression is caused by toxins (like bacterial endotoxins and mycotoxins), nutritional deficiencies, and infectious agents. Although lymphocyte repopulation occurs, tissues such as the caecal tonsil may have permanent depletion of diffuse lymphocytes in the lamina propria. Lymphocytic follicles are reduced in number if a young bird is exposed when seeding of secondary lymphoid tissues occurs from the bursa of Fabricius (HOERR et al. 1982a, b). The immunsupression caused by toxins produces a cascading effect starting with decreased protein synthesis, lowered serum albumin and globulin levels, reduction in circulating antibody levels, impairment of the reticulo-endothelial system and reduced cell-mediated immunity, besides affecting the normal development of the bursa of Fabricious (DEVEGOWDA et al. 1995).

Reduction in the relative size of the bursa of Fabricius accompanied by mortality due to aflatoxin has been observed by THAXTON et al. (1974), KUBENA et al. (1990). THAXTON et al. reported the atrophy of thymus and bursa of Fabricius on feeding aflatoxin-contaminated diets to chicken (THAXTON et al. 1974). RAJU and DEVEGOWDA observed a significant reduction in the size of the BF in broilers fed diets containing 500 ppb aflatoxin (RAJU and DEVEGOWDA 2000). VIRDI et al. observed thymic aplasia and reduced weight of the bursa of Fabricius up to the extent of 38 in chicken fed aflatoxin-contaminated diets (VIRDI et al. 1989).

5.2 Discussion of results

5.2.1 Effect of inulin (0.5% via drinking water) and food supplement with 1% linseed on the naturally colonization of young chicks with *S.* Enteritidis

Bacterial diseases remain of major economic and public health importance. That perhaps comes most readily to mind with the novel approaches that will be explored. In recent years, the animal and public health problems associated with *Salmonella* in poultry have increased to the extent that they have become major political issues of which the general public in several countries have been aware. *S.* Enteritidis, in particular, has become a world-wide problem (RODRIGUE et al. 1990).

In many countries individual phage types of this serotype have replaced *S.* Typhimurium as the predominant type in poultry and man. Infection control has thus become a major issue. However, it must be remembered that although, in some countries, the problem of *S.* Gallinarum and *S.* Pullorum infections has been virtually eliminated by a policy of serological test and slaughter, they remain major, and in some eases increasingly important problems in many countries.

In recent years, *S.* Enteritidis has become the predominant *Salmonella* causing human salmonellosis in western countries such as the United States and The Netherlands (GAST 1999). The main reservoirs for human serovar Enteritidis infections are poultry and poultry products (VAN DE GIESSEN 1999). Infection of broiler chicken with serovar Enteritidis is age and dose dependent (GAST 1999, GAST and BENSON 1996, GORHAM et al. 1991). Young broiler chickens are particularly susceptible to *Salmonella* infections (GORHAM et al. 1991).

In our studies, we have examined cloacal swabs from thirty young chicks (one-day-old) and all birds were positive to *S.* Enteritidis. The addition of inulin to the drinking water (I-group) or inulin and linseed to these young chicks (IL-group) reduced the incidence of a naturally intestinal colonization with *S.* Enteritidis. After the first week of treatment, the *Salmonella* colonization of the gut was reduced from 100% to 80% in the I-group and to 60% in the IL-group, whereas there were no changes in the *Salmonella* colonization in control animals (C-group). In the 2nd week of the experiment the *Salmonella* colonization was 80%, 60% and 40% in C, I and IL-groups respectively. At the 3rd week of the experiment both I and IL-groups (3, 4) were negative to *Salmonella* colonization and in C-group the colonization was reduced to 40%. The results indicated that resistance to *S.* Enteritidis colonization may be effectively increased in young chicks by the addition

of inulin to the drinking water (I-group) or combination of inulin (via drinking water) and linseed supplementation 1% to the ration (IL-group).

NURMI and RANTALE (1973) were the first to suggest that this susceptible period is caused by the lack of a mature microflora in young broilers. They observed that treating 1-day-old broilers with a mature caecal microflora from *Salmonella*-free adult chicken protected these broilers from colonization with *Salmonella* in the caeca. The exact mechanism behind reduction of *Salmonella* numbers by the mature caecal microflora is not known. Several mechanisms have been postulated: competition for nutrients, competition for receptor sites, immunomodulation, production of antimicrobial substances, or production of volatile fatty acids, such as acetate, propionate, and butyrate (CORRIER et al. 1990). Perhaps the most important function of the indigenous intestinal microflora to the host is its ability to inhibit the colonization of invading pathogens in the intestinal tract. Many complex bacterial control mechanisms are involved in regulating the composition of the gut microflora and in excluding intestinal pathogens. Imbalances in the gastrointestinal ecosystem can weaken the protective effect of the indigenous microflora which gives enteric pathogen a better chance to colonize in the gut. Imbalances in the ecosystem mainly occur in the young animal, during periods of stress, changes in the periods of nutritive application and/ or digestive disorders. The young bird combines all these situations. It therefore not surprise that the young bird is prone to the colonization with enteric pathogens such as salmonellae (ROLFE 1991). In our results we have found that the newly hatched chick is very susceptible to *Salmonella* infections, the colonization index was 100% in all examined groups. In the second week of the experiment the colonization was 80% in C-groups and at the third week the colonization was reduced to 40%. This indicates that as the chick ages its resistance to *Salmonella* infection increased. The same findings were reported by ZIPRIN et al. (1989). They have found that the newly hatched chick is very susceptible to *Salmonella* infections, but its resistance increases with age. They suggested that increasing resistance to *Salmonella* infections could be attributed to development of a competent T cell-dependent immune system and to the acquired resistance through colonization of the intestinal tract of beneficial microflora.

Young broiler chickens (1 to 14 days old) do not contain anaerobic bacteria as a dominant fraction of their caecal microflora. Therefore, the concentrations of acetate, propionate, and butyrate are low in the caeca during the first week of life. In vivo, it was observed that the increase in concentrations of acetate, propionate, and butyrate in the caeca is a cause for the decrease in viable counts of members of the family *Enterobacteriaceae* in the caeca of broiler chicken (VAN DER WIELEN et al. 2000).

GAST and BEARD (1989) found that the birds inoculated at 1 day posthatch had more mortality, morbidity and greater numbers of *S.* Typhimurium cfu at 7 week than these chicks inoculated orally between 1 and 8 day posthatch, but the pathogenic organism persisted in the caeca of the 7-week-

old broilers without any mortalities. Furthermore, *S.* Typhimurium adhered to the epithelium of the caeca in the birds inoculated at 1 day more readily than in the birds inoculated at 3, 5, or 7 day post-hatch, but age did not affect the recovery of the organism from the spleen or the liver. These observations by GAST and BEARD (1989) strongly suggest that a symbiotic relationship between the avian host and protective microflora in the intestinal tract develops after hatch so that *S.* Typhimurium inoculation after 1 day posthatch was less severe. Additionally, the age-related decrease in mortality further indicated that some kind of adaptation or developed resistance had occurred. SOERJADI et al. (1981a, b) and STAVRIC et al. (1991) have shown that resistance to *Salmonella* is developed only when there is colonization of the intestinal tract with beneficial microflora.

Dietary inputs can beneficially affect the host animal by improving its intestinal microbial balance (COLLINS and GIBSON 1999). However the influence of diet is complex, they involve several possible mechanisms of action. Dietary inputs can serve as substrates for the bacteria. Some components of the diet can alter the assemblages of bacteria, just as adding fermentable fibers, such as oligofructose and inulin, selectively increase the abundance of lactic acid bacteria while decreasing the percentages of potential pathogens and putrefactive bacteria. They also influence the metabolic activities of bacteria. They also increased resistance to infectious diseases, particularly of the intestine, decreased duration of diarrhoea, reduction in serum cholesterol concentration, stimulation of phagocytosis by peripheral blood leucocytes, modulation of cytokine gene expression, whereas many antibiotics suppress the healthy population of intestinal flora resulting in "dysbiosis" which leads to enteritis and disturb the GIT bacterial assemblages and this can affect the structure and functions of the mucosa. The changes in the microenviroment can lead to the proliferation of some pathogens, such as clostridia (BUDDINGTON and WEIHER 1999).

An alternative approach to control *Salmonella* during grow-out has been the addition of carbohydrates to the diet of chicken. OYOFO et al. (1989) found that mannose and lactose significantly reduced the intestinal colonization of *S.* Typhimurium by at least one-half, as compared with dextrose, maltose, and sucrose. Lactose and mannose also significantly reduced the mean $\log_{10}$ number of *S.* Typhimurium in the caecal contents. Lactose does not offer protection against *S.* Typhimurium by promoting the growth of lactobacilli alone. It is likely that lactose produces VFs toxic to *S.* Typhimurium and also promotes the growth of lactobacilli that either competes with *S.* Typhimurium for colonization sites.

S. Typhimurium colonization, pH and density of the caeca was measured in 3-, 5-, and 6-week-old broilers fed either a control ration or rations with added refined fructooligosaccharides (FOS) or lactose derivatives (LD). Chicks were challenged commencing at 5 d, by exposure to chicks orally infected with *S.* Typhimurium. The high prevalence of *Salmonella* infection at 3 wk declined as chicks aged. The decline of *Salmonella* infection of broilers fed either refined FOS or LD ceased after dietary additives were discontinued at 5 week of age. At 6 week, infection rates of the latter

groups were at least as high as those of control broilers. Both FOS and LD reduced caecal pH and density, (CHAMBERS et al. 1997).

There are several published studies testing the effects of either lactose (Corrier et al. 1990a, b, 1991, DELOACH et al. 1990, HINTON et al. 1990, NISBET et al. 1993, TELLEZ et al. 1993) or fructooligosaccharides (FOS) (BAILEY et al. 1991, CHOI et al. 1994, CONNER and MORAN 1992, MORAN and CONNER 1992, WALDROUP et al. 1993) in the diet for ability to control *Salmonella* in poultry. Modest reductions of caecal *Salmonella* counts or caecal *Salmonella* colonization associated with dietary lactose or lactose-containing products have been reported (DELOACH et al. 1990, CORRIER et al. 1991, HINTON et al. 1990); however, NISBET et al. (1994) observed little reduction of *Salmonella* colonization of 10-day-old broilers fed dietary lactose. CORRIER et al. (1990a, b) reported that dietary lactose increased caecal acidity and influenced intestinal flora involved in the control of enteropathogens in poultry. *Salmonella* control was believed to be achieved by stasis caused by low pH or elevated levels of volatile fatty acids. Dietary FOS did little to reduce *Salmonella* colonization of caecal contents of broiler chicks (BAILEY et al. 1991, CONNER and MORAN 1992) or contamination of broiler carcasses (Waldroup et al. 1993). However, in one study, dietary FOS reduced caecal colonization by *S.* Typhimurium as well as counts of *S.* Typhimurium of colonized broiler chicks (CHOI et al. 1994). Dietary lactose (CORRIER et al. 1990a, b, DELOACH et al. 1990, HINTON et al. 1990) and FOS (CHOI et al. 1994, MORAN and CONNER 1992) have been associated with reductions in caecal pH. A trend for reduced caecal pH associated with elevated lactic acid levels and reduced *Salmonella* counts were apparent in some studies (CORRIER et al. 1990a, b, HINTON et al. 1990).

Prebiotics induce changes in the population and metabolic characteristics of the gastrointestinal bacteria, modulate enteric and systemic immune functions, and provide laboratory rodents with resistance to carcinogens that promote colorectal cancer. Mice fed the diets with fructans had 50% lower densities of *C. albicans* in the small intestine (BUDDINGTON et al. 2002).

Inulin is a carbohydrate belonging to a class of compounds known as fructans. Because inulin is resistant to digestion in the upper gastrointestinal tract it reaches the large intestine essentially intact, where it is fermented by indigenous bacteria, thus, it may be classified as a soluble dietary fiber. Soluble fibers have been shown to modulate serum lipids. Inulin-type fructans are indigestible oligosaccharides for which a wide range of scientific observations are already available and which demonstrate an array of potential health benefits. Inulin and oligofructose are present as plant storage carbohydrates in a number of vegetables and plants including wheat, onion, bananas, garlic and chicory. Inulin induce changes in the composition of the colonic microbiota, modulation of the metabolism of triacylglycerol, modulation of insulinemia, improved bioavailability of dietary calcium. In addition to having nutritional properties, which may justify their classification as functional food (ROBERFROID 2000).

Dietary incorporation of fermentable, indigestible fructans (Oligofructose and inulin) may be of benefit to gastrointestinal health by providing short-chain fatty acids, stimulating the proliferation of bifidobacteria or lactobacilli and suppressing potential pathogenic (KLEESSEN et al. 2001). Inulin and oligofructose are rapidly and completely fermented by the colonic microflora with the production of acetate and other short-chain fatty acids. They may also result in the growth of the faecal biomass; entrap ammonia for bacterial protein synthesis or conversion to the ammonium ion (JENKINS et al. 1999).

Our results agree with results of other investigators they found suppression of pathogenic bacteria like *Salmonella* spp. or others after feeding of fructooligosaccharides (Miles 1997). BAILEY et al. (1991) showed that in birds stressed by feed and water deprivation, more *Salmonella*-positive birds were found in control birds than those fed fructooligosaccharide. Thus, oligosaccharides may be beneficial when animals are raised under less than ideal conditions. Studies reporting the use of fructooligosaccharides in poultry diets have indicated improvements in weight gain and feed efficiency, reduction in mortality, and reduction in intestinal colonization by *Salmonella* (AMMERMAN et al. 1988, 1989, FARNWORTH et al. 1993, WALDEROUP et al. 1993).

In practical terms, when FOS is fed to chicken, the glucose and fructose residues are absorbed in the upper part of the intestinal tract (HUME et al. 1993), and that intact FOS molecules reach the lower part of the GI tract, especially the caeca, where they serve as a growth promotant for those bacteria with biochemical capabilities to utilize FOS. It has been demonstrated that FOS can enhance the activity of competitive exclusion cultures when co-fed to broiler chicks (BAILEY et al. 1991).

Flaxseed (Linseed) considered as functional food for people and other animals. It contains large amounts of OMEGA 3 fatty acid, alpha linolenic acid an essential fatty acid (EFA), that animal bodies can't make from other foods and very high amounts of soluble and insoluble dietary fiber and other nutrients such as proteins, carbohydrates, minerals. Flaxseed is very high in potassium. EFAs are not made by a birds' body and must be supplied daily through food supplement. The principal precursor of eisosanoids in poultry (WATKINS 1991) is arachidonic acid (AA, C20:4n-6). Eicosanoids are the substrates of several oxidative metabolic pathways for the production of prostaglandins (PG), and leukotrienes (LT). The PG and LT are involved in various immune responses. Prostaglandin E_2 modulates the production of interleukin (IL)-l and tumour necrosis factor (TNF). However, overproduction of PGE_2 (KINSELLA et al. 1990), or high dosages of n-6 polyunsaturated fatty acids (PUFA) can also suppress immune responses in vivo (ERICKSON et al. 1983).

The effect of linoleic and linolenic acid on antibody (Ab) responses to sheep red blood cells (SRBC) and bovine serum albumin (BSA) and on growth performance were studied in pullets of three genetically different laying lines. Pullets were fed one of three diets: a control diet, a linoleic acid-enriched diet, or a linolenic acid enriched-diet. The linoleic and linolenic acid enriched-diets

were enriched with either sunflower oil or linseed oil. Total Ab responses to SRBC were not affected by diet, but a tendency for higher IgG titers to SRBC after primary immunization were found when birds were fed the linoleic diet. The humoral response to BSA was significantly affected by diet interaction.The linoleic diet significantly enhanced Ab liters to BSA as compared to the normal diet and linolenic acid-enriched diet. During the first 3 week of life, the linolenic acid-enriched diet resulted in reduced BW gain of H line birds. After that period, no effects of diet on body weight gain were found (PARMENTIER 1997). The requirement of linoleic acid for laying pullets is recommended to be 1% of the diet, but 0.8% may satisfy growth and maintenance requirements (National Research Council 1994). Also, linolenic acid may be required, but the minimal requirement for linolenic acid is unknown. In the current study, diets were not deficient in linolenic acid. Application of dietary essential fatty acids may have potential improvements for flock health and disease resistance, but the effects of dietary fats on immune responses in poultry, as in mammals, are varied (FRIEDMAN and SKLAN 1995). Diets high in n-3 PUFA reduced lesion scores in young broilers infected with *Eimeria tenella* (ALLEN et al. 1996). At the present time there are no official guidelines on essential fatty acid intake for poultry. It is recognized that EFAs can reduce infection, improve immune status and reduce inflammation. EFAs are a very important part of a stress reduction program. Flaxseed favourably influences immune response. The flaxseed component, alpha linolenic acid, alters membrane phospholipids, inhibits arachidonic acid biothsynthesis from linoleic acid, and inhibits the production of proinflammmtory eicosanoids from arachidionic acid (LEAF and WEBER 1988).

The alimentary tract of the newly hatched, healthy chicken is usually sterile (MEAD and ADAMS 1975, SAVAGE 1987). Soon after hatching, young birds naturally develop a mature intestinal microflora through contamination with a mature intestinal microflora through contamination with faecal material from mature birds. With corprophagic birds, such as the domestic fowl, the transfer of bacteria from parent to chick occurs very efficiently and allows the young animal to establish a protective intestinal flora within the first couple of days after hatching (SPRING 1997). Recent advances in biosciences support the hypothesis that diet modulates various body functions. Diet may maintain well-being and reduce the risk of some diseases. Such discoveries have led to the concept of "functional food" and the development of the new discipline, i.e., "functional food science". Functional food ingredients" are the inulin-type fructans, i.e., chicory inulin and oligofuctose and linseed have effects on the intestinal microflora, the gastrointestinal physiology, the immune functions, the bioavailability of minerals and the metabolism of lipids. Growth of natural microflora able of inhibiting enteropathogen growth in the chicken gastrointestinal tract may be promoted by addition of dietary supplementation of inulin and linseed.

5.2.2 The effect of inulin and linseed on some intestinal flora, BW and on immune status of SPF chicks

In our studies we have found that, there was a significant decrease in total aerobic bacterial counts in the small intestine and caecum only but not in the crop and rectum of the IL-group and I-group at the 3rd week of the trial in comparison with control birds, gram-negative bacterial count also was significantly decreased in crop and small intestine of IL-group and I-group at the 2nd week and in the caecum and rectum at the 3rd week but not in the C-group. *Bdellovibrio bacteriovorus* counts were significantly increase only in the caecum and rectum of the IL-group and I-group respectively at the 2nd week but can not be isolated from the C-group.

Our results agree with RAO (1999) he found the daily ingestion of inulin stimulate the growth of bifidobacteria in humans, whereas the number of bacteriodes, coliforms and gram-positive cocci decreased. Also our results agree with ORBAN et al. (1997) studied the effect of oligosaccharides on the growth and intestinal populations of broiler chicken. He found that, the feeding of oligosaccharides significantly decreased total aerobes and coliforms, but had little effect on either aerobically or anaerobically enumerated lactobacilli or on bifidobacterial numbers in the caecum.

In our results, there was no significant difference in body weight between all groups but there was a significant increase in BF/BW and P/BW ratios in the I- group and in the IL-group than in C-group indicated increase immune capacity of IL-group and I-group. Provision of carbohydrates in the drinking water had no significant effect on weight gain. In the intestinal tract of weaned animals lactose is less readily digested than other sugars such as glucose, galactose, sucrose and maltose (ATKINSON et al. 1957).

Also GIBSON (1999) found that the use of oligofructose or inulin in human diet caused a marked increase in bifidobacteria, whereas bacteriods, fusobacteria and clostridia all decreased.

Feeding fructooligosaccharides have been shown to improve animal performance, reduce serum cholesterol, reduce disease-related diarrhoea, alleviate constipation, reduce intestinal concentrations of putrefactive compounds, reduce tumours, and enhance the immune response in a number of species (HIDAKA et al. 1986). Immune function enhancement has been proposed as a possible function of inulin, oligofructose and lactulose. Studies have not been undertaken to assess systematically lymphocyte activity. However inulin, oligofructose and lactulose administration raise serum glutamine. Because glutamine is a preferred substrate for lymphatic tissue, it is possible that this may be improving immune function under some circumstances (JENKINS et al. 1999).

5.2.3 The effect of inulin on the intestinal flora, endotoxin and phosphoryl choline-binding protein (PC-BP) blood levels and on the immune status of broiler chicken

There was no significant difference in total aerobic bacterial count in the caecum of I-group (with inulin) and C-group (without inulin) after one week of inulin administration but at the second week

the total bacterial count by I-group was significantly decreased than C-group. Gram-negative bacterial counts were also significantly decreased after 2 weeks of inulin administration by I-group. Whereas the *Bdellovibrio* counts were slightly increased in the I-group after one week of inulin administration. At the second week and until the end of the experiment (5 weeks) the *Bdellovibrio* counts were significantly increased than C-group. On the other hand, there was a decrease in the *C. perfringens* caecal counts of I-group, which were slightly significant at the first week of the experiment. At the third week there was a strongly significant decrease in *C. perfringens* caecal counts of the I-group in comparison with C-group. There was also a negative correlation between *C. perfringens* and *Bdellovibrio* caecal count. There were no significant changes in body weight between I-group (with inulin) and C-group (without inulin) during the experiment but at the 5th week, there was a significant increase in the body weight of I-group, whereas there was a significant increase in BF/BW ratio in the I-group after 2 weeks of inulin administration until the end of the experiment.

In similar studies (CATALA 1999) found that oligofructose significantly increased the level of bifidobacteria and this was associated with a decrease of *E. coli* or *C. perfringens* and C. ramosum. It was demonstrated that, irrespective of the environmental conditions, the use of oligofructose helped to prevent the overgrowth of bacteria implicated in necrotising enterocolitis in preterm neonates. The changes in microflora balance may due to the production of bacterial metabolite, i.e., short-chain fatty acids and hydrogen. Bifidobacteria are thought to exert various beneficial effects on host health, including interaction with the colonic microflora. Therefore, it was hypothesised that a protective role could be exercised through bifidobacterial colonization.

MORISHITA et al. (1982) found that, a decrease in the incidence of clostridia in the caecal contents of chicks fed on a purified diet containing 300 g lactose plus 300 g starch/kg when compared with that of control birds receiving a diet containing 600 g starch/kg. High counts of Proteus spp. were present in the caeca of control birds but they were completely suppressed in conventional birds fed on the lactose dietIn vitro tests showed that this inhibition was partially due to *E. coli* and *E. faecalis.* On the same manner, the in vitro fermentability of oligofructose and inulin studies showed that oligofructose and inulin exerted a preferential stimulatory effect on numbers of the health-promoting genus *Bifidobacterium*, whilst maintaining populations of potential pathogens (*E. coli, Clostridium*) at relatively low levels (WANG and GIBSON 1993). These findings agree with our results, in vivo fermentability studies of inulin on broiler chicken and on the naturally infected chicken with *S.* Enteritidis, we have found an inhibitory effect towards coliforms (gram-negative bacteria) and *C. perfringens*. On the other hand there were stimulatory effects on the *Bdellovibrio* spp. count and on the immune status of the birds. Also GIBSON (1999) found that the use of oligofructose or inulin in human diet caused a marked increase in bifidobacteria, whereas bacteriodes, fusobacteria and clostridia all decreased. The in vitro fermentability of oligofructose and inulin was compared with a range of reference carbohydrates by measuring bacterial end-

product formation in batch culture. Short chain fatty acid and gas formation indicated that these substrates, which occur naturally in the diet and reach the colon in a largely intact form, were utilized by mixed populations of gut bacteria. Bacterial growth data showed that oligofructose and inulin exerted a preferential stimulatory effect on numbers of the health-promoting genus *Bifidobacteriu*m, whilst maintaining populations of potential pathogens (*E. coli, Clostridium*) at relatively low levels. *Bifidobacterium* was able to exert an inhibitory effect not necessarily related to acid production.

Further studies showed that eight species of bifidobacteria could variously excrete an anti-microbial substance with a broad spectrum of activity. Species belonging to the genera *Salmonella*, *Listeria, Campylobacter* and *Shigella*, as well as *Vibrio cholerae*, were all affected. Bifidobacteria are able to exert more than one mechanism of inhibition, which may be of some importance with regard to protection against gastroenteritis. Pure culture studies confirmed the enhanced ability of bifidobacteria to utilize these substrates in comparison with glucose. Batch culture experiments demonstrated that the growth of *Bifidobacterium* Infantis had an inhibitory effect towards *E. coli* and *C. perfringens*. Potentially, an increase in the concentration of these substrates in the diet may therefore improve the composition of the large intestinal microflora and have positive effects on the quality of the diet, (WANG and GIBSON 1993, 1994).

TERADA et al. (1994) reported that feeding lactosucrose to broilers increased numbers of bifidobacteria, decreased concentration of putrefactive products (phenol and p-cresol) and ammonia, and increased the concentration of volatile fatty acids in broiler faeces. Thus, oligosaccharides may potentially be useful in reducing ammonia and other environmental odours emanating from poultry production facilities. Bifidobacteria are recognised to have beneficial effects on digestive disorders.

Prebiotics escape enzymatic digestion in the upper gastrointestinal tract and enter the caecum without change to their structure. None are excreted in the stools, indicating that they are fermented by colonic flora so as to give a mixture of short-chain fatty acids (acetate, propionate and butyrate), L-lactate, carbon dioxide and hydrogen. By stimulating bifidobacteria, they may have the following implications for health: inhibition of bacterial enteritis (diarrhea) by inhibiting putrefactive bacteria (*C. perfringens*) and pathogenic bacteria (*E. coli*, *Salmonella*, *Listeria* and *Shigella*), respectively, improvement of lipid metabolism, fibre-like properties by decreasing the renal nitrogen excretion, improvement in the bioavailability of essential minerals, in particular, calcium, and stimulation of the immune system (GRIZARD and BARTHOMEUF 1999, ROBERFROID 1999). Oligofructanes are able to bind lectins and prevent attachment of bacteria to epithelial cells. Oligofructanes like inulin are able to promote the indigenous microflora of the gastrointestinal tract. This microflora is able to promote the mucosal associated immunity by production of bacterial peptides. These peptides for instance from *Propionibacterium acnes, S. saprophyticus, Bacteroides* spp.,

Propionibacterium spp. stimulate immatural lymphoid cells to maturation (BEUTH et al. 1992). The most *Bifidobacterium* spp., *Eubacteriaceae*, a lot of *Bacteroides* strains are able to hydrolyze fructooligosaccharides. These bacteria are promoted. The number of these increases and influence cocci and clostridia. Inulin influences the production of butyric acid in a positiv manner. More than 90 % of butyric acid is absorbed in caecum and colon and is used as the most important energy source of enterocytes. Inulin also influences the lipid pathway by reducing lipoproteins, especially the LDL and VLDL fractions. HDL is only few influenced (DE VRESE 1997, MC BAIN and MAC FARLANE 1997, SOBOTKA et al. 1997).

CORRIER et al. (1990) found that, there were no significant differences in the body weights of the chicks provided lactose compared with the chicks in control group. These findings agree with our results, by adding inulin to the experimental chicks there were no significant differences in the body weights. It was accepted, that endotoxin level in the blood of I-group was significantly reduced in comparison with control birds especially at the end of the experiment at 6^{th} week. There was also a significant reduction in phosphoryl choline binding protein (PC-BP) at the second week and at the 5^{th} week in the blood of the I-group in comparison with control birds. There was negative correlation between PC-BP, endotoxin levels in the blood of birds and growth rate of body weight. Also at increasing total aerobic, gram-negative and clostridial bacterial counts there were an adverse effect on the body weight, whereas there was a positive correlation between *Bdellovibrio* count and growth rate of body weight of birds.

Breakdown of both the mucosal barrier and the reticuloendothelial system (RES) capacity results in systemic endotomia. Systemic endotoxaemia results in organ dysfunction, impairs the mucosal barrier, the clotting system, the immune system, and depresses Kupffer cell function. If natural defence mechanisms such as lipopolysaccharide binding protein, high density lipoprotein, in combination with the RES, do not respond properly, dysfunction of the gut barrier results in bacterial translocation. The gastrointestinal tract, besides being the organ responsible for nutrient absorption, is also a metabolic and immunological system, functioning as an effective barrier against endotoxin and bacteria in the intestinal lumen. The passage of viable bacteria from the gastrointestinal tract through the epithelial mucosa is called bacterial translocation. Equally important may be the passage of bacterial endotoxin through the mucosal barrier (VAN LEEUWEN 1994). Selective decontamination of *Enterobacteriaceae* from the digestive tract did cause a significant decrease in the faecal endotoxin concentration in a subset of patients treated for Selective decontamination, (VAN DER WAAIJ et al. 1985). In our studies inulin significantly increased the *Bellovibrio* counts and this was associated with a decrease of total aerobic and gram-negative and *C. perfringens* bacterial counts. This explains the significant decrease of endotoxin and PC-BP levels in the blood of I-group.

5.2.4 Study of the dynamic of some intestinal flora of broilers and breeders chicken in relation to endotoxin and PC-BP blood levels, BF/BW and P/BW ratios

We have found a conversely relationship between *Bdellovibrio* and *C. perfringens* counts. The *Bdellovibrio* counts were negatively related to total aerobic, gram-negative bacterial counts and endotoxin blood levels. On the other hand *Bdellovibrio* caecal counts were positively related with the immune ratios (BF/BW and P/BW ratios) and to body weight, whereas the increase in the endotoxin and PC-BP blood levels had negative effect on these immune ratios. The endotoxin blood level was also found positively related to PC-BP level and to increasing in the total aerobic and gram-negative bacterial counts. P/BW ratio was positively related with BF/BW ratio. The endotoxin blood level was also found positively related to PC-BP.

MURSATA et al. (1965) and FUKATA et al. (1991) found that germ free chicken were more sensitive to *C. perfringens* or to its toxin than conventional chicken and this indicates that intestinal flora were responsible, in part, for the decreased sensitivity. It is likely that when changes in intestinal microflora, the number of *C. perfringens* may increase, which in turn results in a greater production of alpha toxin by *C. perfringens*, and outbreak of necrotic enteritis may be induced.

Bdellovibrios, because of their lytic properties, play an important role in maintenance of homeostasis in the ecological system (EDAO et al. 1998). The presence of an indigenous GI microflora like bdellovibrios preventing bacterial overgrowth and this lead to an intact intestinal epithelial barrier, and normal host immune defences. This proves the participation of bdellovibrios in metabolic cycle of nature. The behaviours and the way of life of bdellovibrios (lysis of bacteria) call to attention the question of practical application of these unique microorganisms to fight pathogenic and undesirable gram-negative bacteria. Whether or not bdellovibrios can be applied in the sense of biological control.

6. Summary

Investigations on the significance of the gastrointestinal flora for the immune system of chickens

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(128 pages, 60 figures, 21 tables and 227 references)

Many complex bacterial control mechanisms are involved in regulating the composition of the gut microflora and in excluding intestinal pathogens. Imbalances in the gastrointestinal ecosystem can weaken the protective effect of the indigenous microflora which gives enteric pathogen a better chance to colonize in the gut. Imbalances in the ecosystem mainly occur in the young animal, during periods of stress, changes in the periods of nutritive application and/ or digestive disorders. The young bird combines all these situations. It therefore not surprise that the young bird is prone to the colonization with enteric pathogens such as salmonellae. Understanding the gastrointestinal microflora is essential not only for the development of probiotics but also for developing new prebiotics.

1- Effect of inulin 0.5% via drinking water and feed supplement with 10% linseed on the natural colonization of young chicks with *S.* Enteritidis was examined; the addition of inulin to the drinking water (I-group) or inulin and linseed to the young chicks (IL-group) reduced the incidence of a naturally intestinal colonization with *S.* Enteritidis.

2- Effect of inulin 0.5 % via drinking water and food supplemented with 1% linseed on the intestinal bacterial population and the immune status of SPF chicken was investigated, the bacteriological examinations revealed a significant decrease in total aerobic bacterial count in the small intestine and caecum only but not in the crop and rectum of the IL-group and I-group respectively at the third week of the trial in comparison with control birds. Gram-negative bacterial counts also were significantly decreased in crop and small intestine of IL-group and I-group at the second week and in the caecum and rectum at the third week but not in the C-group. *Bdellovibrio bacteriovorus* counts were significantly increase only in the caecum and rectum of the IL-group and I-group respectively at the second week but can not be isolated from the C-group. There was no important difference in BW between all groups but there was a significant increase in BF/BW ratio in the I- group and in the IL-group than in C-group indicated increase immune capacity of IL-group and I-group.

3- The effect of inulin on the intestinal flora, endotoxin and PC-BP blood levels and on the immune status of broiler chicken was investigated**,** There was no significant difference in the total aerobic bacterial counts in the caecum of I-group (with inulin) and C-group (without inulin) after one week of inulin administration but at the second week the total bacterial count by I-group was significantly decreased than in C-group. Gram-negative bacterial counts were also significantly decreased after 2 weeks of inulin administration by I-group, whereas the *Bdellovibrio* counts were slightly increased in I-group after one week of inulin administration. At the second week and until

the end of the experiment (5 weeks) the *Bdellovibrio* counts were significantly increased in I-group than in C-group. On the other hand, there was a decrease in the *C. perfringens* caecal counts of I-group, which were slightly significant at the first week of the experiment. At the third week there was a strongly significant decrease in *C. perfringens* caecal counts of the I-group in comparison with C-group. There was also a negative correlation between *C. perfringens* and *Bdellovibrio* caecal counts. There were no significant changes in BW between I-group (with inulin) and C-group (without inulin) during the experiment but at the 5^{th} week, there was a significant increase in the BW of I-group, whereas there was a significant increase in BF/BW ratio in the I-group after 2 weeks of inulin administration until the end of the experiment. The endotoxin level in the blood of I-group was significantly reduced in comparison with control birds especially at the end of the experiment at 6^{th} week. There was also a significant reduction in phosphoryl choline binding protein (PC-BP) at the second week and at the 5^{th} week in the blood of the I-group in comparison with control birds. The PC-BP, endotoxin levels in the blood of birds were negatively related to the growth rates of BW. Also at increasing total aerobic, gram-negative and clostridial bacterial counts there were an adverse effect on the BW, whereas there was a positive correlation between *Bdellovibrio* count and growth rate of BW of birds.

4- When studying the dynamic of normal caecal flora of young (broilers) and adult (breeders) chicken we have found a conversely relationship between *Bdellovibrio* and *C. perfringens* counts. The *Bdellovibrio* count was negatively related to total aerobic, gram-negative bacterial counts and endotoxin blood level. On the other hand *Bdellovibrio* caecal counts were positively related with the BF/BW and P/BW ratios and to body weight, whereas the increase in the endotoxin and PC-BP blood levels had negative effect on these immune ratios. The endotoxin blood levels were also found positively related to phosphoryl choline-binding protein (PC-BP) levels and to increasing in the total aerobic and gram-negative bacterial counts. P/BW ratio was positively related with BF/BW ratio. Results of the present studies indicate that inulin offer protection to chickens by:

1- Markedly decreased intestinal colonization by *C. perfringens* counts.

2- Increase in *Bdellovibrio bacteriovorus* counts.

3- Decrease in the endotoxin level of blood.

4- Decrease in the PC-BP level of blood.

5- Enhancing the immune status of the bird.

From our results and previous studies, we can conclude that inulin and linseed can be classified as functional diat in poultry, because they reduce the risk of some diseases (like *Salmonella*). Inulin and linseed have enhanced effects on the intestinal microflora and on the immune status of birds and play important role in the maintenance of homeostasis in the ecological system. Additional research is required in order to determine the effect of dietary inulin on the other important intestinal flora of chicken like *Lactobacillus* and bifidobacteria. Also the combination of inulin with probiotic (synobiotic) and their role in enhancing the local immunity of gastrointestinal tract and the competitive exclusion against pathogenic bacteria like *Salmonella*.

7. Zusammenfassung

Untersuchungen zur Bedeutung der Magen-Darm-Flora auf das Immunsystem von Hühnern

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Viele komplexe bakterielle Kontrollmechanismen der Darmmikroflora sind am Ausschluss pathogener Keime beteiligt. Dysregulationen im gastrointestinalen Ökosystem können den schützenden Effekt der indigenen Mikroflora schwächen, was die Kolonisation von pathogenen Keimen im Darmkanal begünstigt. Dysregulationen im gastrointestinalen Ökosystem treten bei Jungtieren hauptsächlich unter Stress, bei der Futterumstellung oder Verdauungsstörungen in Erscheinung. Alle diese Situationen kommen bei Jungvögeln vor. Dem zu Folge ist es nicht überraschend, dass Jungvögel empfindlicher für die Kolonisation von pathogenen Darmerregern wie Salmonellen sind. Das Verstehen der gastrointestinalen Mikroflora ist nicht nur für die Entwicklung von Probiotika sondern auch für das Entwickeln neuer Präbiotika wichtig.

1. Effekt von 0.5% Inulin im Trinkwasser und 1% Leinsamen im Futter auf die natürliche Besiedlung junger Küken mit *S.* Enteriditis, die Zugaben von Inulin im Trinkwasser (I-Gruppe) oder Inulin und Leinsamen (IL-Gruppe) an die jungen Küken führte zur Reduzierung der Nachweisrate von *S.* Enteritidis bei natürlich besiedelten Küken.

2. Effekte von 0.5% Inulin im Trinkwasser und 1% Leinsamen im Futter auf die intestinale bakterielle Besiedlung und den Immunstatus von SPF Hühnern, die bakteriologischen Untersuchungen offenbarten einen signifikanten Abfall der aeroben Gesamtbakterienzahl im Dünndarm und in Zäkum, aber nicht in Kropf und Rektum, insbesondere von der dritten Versuchswoche in den IL- und I-Gruppen gegenüber den Kontrolltieren. Die Zahl der gramnegativen Bakterien war in der zweiten Woche im Kropf und Dünndarm in den IL- und I-Gruppen deutlich rückläufig sowie in der dritten Woche im Zäkum und Rektum aber nicht in der C-Gruppe.
Die Bdellovibrionenzahl stieg signifikant nur im Zäkum und im Rektum der IL- und I-Gruppen an, dem gegenüber konnten in der C-Gruppe keine Bdellovibrionen isoliert werden. Bedeutsame Unterschiede der Körpergewichte zwischen all den Gruppen zeigten sich nicht, wobei das signifikante Ansteigen die Quotient aus Bursa Fabricii/Körpergewicht in den IL- und I-Gruppen gegenüber der C-Gruppe auffielen.

3. Effekte von Inulin auf die Darmflora, den Endotoxin- und PC-BP-Blutspiegel und den Immunstatus von Broilern, nach der ersten Woche der Inulin-Verabreichung zeigten sich kaum

Unterschiede in den Gesamtzahlen der aeroben Bakterien im Zäkum in der I-Gruppe (mit Inulin) und C-Gruppe (ohne Inulin). Hingegen stieg die Gesamtkeimzahl in der I-Gruppe gegenüber der C-Gruppe deutlich an. Gramnegative Bakterien fielen zahlenmäßig nach 2 Wochen deutlich bei der I-Gruppe ab. Die Bdellovibrionen steigen nach einer Woche in dieser I-Gruppe etwas an. Ab der zweiten Woche bis zum Ende des Versuchs (also nach 5 Wochen) stieg die Bdellovibrionenzahl in der I-Gruppe signifikant gegenüber der C-Gruppe.
Dem gegenüber fiel die *C. perfringens*-Zahl in dieser I-Gruppe ab, welches in der ersten Versuchswoche von geringer Signifikanz war. In der dritten Woche fielen die zäkalen *C. perfringens*-Zahlen deutlich in der I-Gruppe gegenüber der C-Gruppe ab, wobei die Clostridien negativ zu den Bdellovibrionen im Zäkum korrelierten. Die Entwicklungen der Körpergewichte im Vergleich der beiden Gruppen zeigten insbesondere ab der 5.Woche signifikante Vorteile in der I-Gruppe. Die Quotienten aus Bursa Fabricii/Körpergewicht und Pankreas/Körpergewicht nahmen deutlich 2 Wochen nach der Inulin-Verabreichung bis zum Versuchsende zu. Die Endotoxin-Blutspiegel reduzierten sich in der I-Gruppe im Vergleich zu den Kontrolltieren besonders deutlich zum Ende des Experiments in der 6. Woche. Ebenso war eine Reduktion des PC-BP-Blutspiegels in der I-Gruppe in der zweiten Woche feststellbar in der 5.Woche. Die PC-BP-und Endotoxin-Blutspiegel korrelierten negativ zu den Körpergewichtzunahmen. Ebenso wirkten sich Zunahmen der aeroben Gesamtkeimzahl, gramnegativen- und Clostridien-Zahlen ungünstig auf die Körpergewichtszunahmen aus. Andererseits zeigt sich hierbei eine positive Korrelation zwischen der Bdellovibrionenzahl und der Körpergewichtzunahme.

4. In den Untersuchungen zur Dynamik der normalen Darmflora im Zäkum der jungen Broiler und erwachsenen Zuchthühner, fanden wir eine umgekehrte Beziehung zwischen dem zahlenmäßigen Vorkommen von Bdellovibrionen und *C. perfringens*. Die Anzahl der Bdellovibrionen korrelierte negativ zu den aeroben Gesamtkeimzahlen, den gramnegativen und *C. perfringens*-Bakterienzahlen sowie Endotoxin- und PC-BP (phosphoryl choline-binding protein)-Blutspiegeln. Andererseits korrelierten sie positiv zu den Quotienten aus Bursa Fabricii/Körpergewicht und Pankreas/ Körpergewicht. Die Endotoxin- und PC-BP-Blutspiegel korrelierten positiv mit dem Anstieg der Gesamtkeimzahlen. Das Pankreas/Körpergewicht-Verhältnis korrelierte positiv zum Verhältnis Bursa Fabricii/Körpergewicht.

Die Resultate der vorliegenden Untersuchungen weisen darauf hin, dass Inulin für Hühner schützende Effekte offeriert:

1) Deutlich abnehmende intestinale Keimzahlen von *C. perfringens.*
2) Anstieg der Bdellovibrionenzahl.
3) Abfall des Endotoxin-Blutspiegels.
4) Abfall des PC-BP-Blutspiegels.
5) Verbesserung des Immunstatus der Vögel.

Aus unseren Resultaten und vorhergehenden Studien können wir feststellen, dass Inulin- und Leinsamen als Funktionelle Futtermittel bei Geflügel eingestuft werden können, weil sie das Risiko einiger Erkrankungen verringern (z.B. durch Salmonellen). Inulin und Leinsamenl besitzen förderliche Effekte auf die intestinale Mikroflora und verbessern den Immunstatus der Tiere. Sie spielen eine wichtige Rolle in der Erhaltung der Homöostase in den ökologischen Systems des Magen-Darm-Traktes.

Weiterführende Untersuchungen wären wünschenswert in Bezug auf die diätetischen Effekte des Inulins auf die anderen wichtigen Vertreter der Darmflora bei Hühnern wie Laktobazillen und Bifidobakterien. Auch die Kombination von Inulin mit Probiotika (Synbiotika) und deren Rolle zur verbesserten lokalen Immunität des Gastrointestinaltrakts und der kompetiven Exclusion pathogener Bakterien wie *Salmonella*-Serovare wären zu untersuchen.

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9. Appendix

Table 1 (A, B) Changes in total aerobic and gram-negative bacterial counts in crop of SPF chicken

A-Total aerobic bacterial count

Age of birds in Weeks	I-group	IL-group	C-group	Significance p≤0.05 between groups
1	(5)* **7.5 ± 1.91***	(5) 7.29 ± 0.85	(5) 8.00 ± 0.76	Not significant
2	(5) 7.4 ± 0.29	(5) 7.2 ± 0.95	(5) 8.47 ± 0.69	Not significant
3	(5) 7.3 ± 1.11	(5) 7.0 ± 0.90	(5) 8.00 ± 0.79	Not significant
Significance p≤0.05 between weeks	Not significant	Not significant	Not significant	

B-Gram-negative count

Age of birds in Weeks	I-group	IL-group	C-group	Significance p≤0.05 between groups
1	(5) 7.0 ± 0.95	(5) 7.0 ± 1.25	(5) 7.20 ± 0.84	Not significant
2	(5) 6.5 ± 1.00	(5) 6.0 ± 1.10	(5) 8.00 ± 0.57	Significant
3	(5) 6.0 ± 0.37	(5) 5.5 ± 0.49	(5) 7.53 ± 0.34	Significant
Significance p≤0.05 between weeks	Not significant	Not significant	Not significant	

Table 2 (A, B) Changes in total and gram-negative bacterial counts in small intestine of SPF chicken

A-Total bacterial count

Age of birds in Weeks	I-group	IL-group	C-group	Significance p≤0.05 between groups
1	(5) 7.92 ± 0.74	(5) 7.0 ± 1.0	(5) 7.99 ± 0.85	Not significant
2	(5) 6.84 ± 1.13	(5) 6.5 ± 0.9	(5) 7.62 ± 1.35	Not significant
3	(5) 5.12 ± 1.00	(5) 5.0 ± 0.49	(5) 8.42 ± 1.38	Significant
Significance p≤0.05 between weeks	Significant	Significant	Not significant	

B-Gram-negative count

Age of birds in Weeks	I-group	IL-group	C-group	Significance p≤0.05 between groups
1	(5) 6.60 ± 0.7	(5) 6.43 ± 0.52	(5) 7.19 ± 0.85	Not significant
2	(5) 6.00 ± 0.63	(5) 5.50 ± 0.63	(5) 7.42 ± 1.37	Significant
3	(5) 5.00 ± 0.31	(5) 5.00 ± 0.31	(5) 7.77 ± 1.82	Significant
Significance p≤0.05 between weeks	Significant	Significant	Not significant	

Table 3 (A, B, C) Changes in total, gram-negative and *Bdellovibrio* bacterial counts in caecum of SPF chicken

A-Total bacterial count

Age of birds in Weeks	I-group	IL-group	C-group	Significance p≤0.05 between groups
1	(5) 8.47 ± 0.46	(5) 7.0 ± 1.25	(5) 8.76 ± 0.85	Not significant
2	(5) 8.02 ± 0.64	(5) 7.0 ± 1.10	(5) 8.03 ± 1.35	Not significant
3	(5) 6.20 ± 1.62	(5) 5.5 ± 0.49	(5) 8.82 ± 1.85	Significant
Significance p≤0.05 between weeks	Significant	Significant	Not significant	

B-Gram-negative count

Age of birds in Weeks	I-group	IL-group	C-group	Significance p≤0.05 between groups
1	(5) 8.22 ± 0.56	(5) 7.0 ± 1.25	(5) 8.41 ± 1.07	Not significant
2	(5) 6.72 ± 0.32	(5) 6.0 ± 1.10	(5) 7.52 ± 1.04	Not significant
3	(5) 5.83 ± 1.35	(5) 5.5 ± 0.49	(5) 8.48 ± 1.98	Significant
Significance p≤0.05 between weeks	Significant	Significant	Not significant	

C-*Bdellovibrio* count

Age of birds in Weeks	I-group	IL-group	C-group	Significance p≤0.05 between groups
1	(5) 0.10±0.00	(5) 0.10±0.00	(5) 0.10±0.00	Not significant
2	(5) 3.00 ± 0.85	(5) 4.8 ± 0.70	(5) 0.10±0.00	Significant
3	(5) 4.30 ± 0.60	(5) 5.0 ± 0.49	(5) 0.10±0.00	Significant
Significance p≤0.05 between weeks	Significant	Significant	Not significant	

Table 4 (A, B, C) Changes in total bacterial, gram-negative und *Bdellovibrio* count in rectum of SPF chicken

A-Total bacterial count

Age of birds in Weeks	I-group	IL-group	C-group	Significance p≤0.05 between groups
1	(5) 8.02 ± 0.47	(5) 7.0 ± 0.45	(5) 8.73 ± 0.56	Not significant
2	(5) 7.75 ± 0.56	(5) 6.5 ± 0.66	(5) 8.52 ± 1.04	Not significant
3	(5) 7.32 ± 0.28	(5) 6.3 ± 0.49	(5) 8.85 ± 1.32	Not significant
Significance p≤0.05 between weeks	Not significant	Not significant	Not significant	

B-Gram-negative count

Age of birds in Weeks	I-group	IL-group	C-group	Significance p≤0.05 between groups
1	(5) 7.70 ± 0.45	(5) 6.99 ± 0.44	(5) 8.40 ± 0.43	Not significant
2	(5) 6.88 ± 1.29	(5) 6.04 ± 0.78	(5) 8.29 ± 1.22	Not significant
3	(5) 6.00 ± 0.44	(5) 5.5 ± 0.49	(5) 8.42 ± 1.43	Significant
Significance p≤0.05 between weeks	Significant	Significant	Not significant	

C-*Bdellovibrio* count

Age of birds in Weeks	I-group	IL-group	C-group	Significance p≤0.05 between groups
1	(5) 0.10±0.00	(5) 0.10±0.00	(5) 0.10±0.00	Not significant
2	(5) 5.04 + 0.44	(5) 3.0 ± 0.29	(5) 0.10±0.00	Significant
3	(5) 3.89 ± 0.56	(5) 3.12 ± 0.52	(5) 0.10±0.00	Significant
Significance p≤0.05 between weeks	Significant	significant	Not significant	

(n)

Statistics mass = x ± s

n^{*} = number of birds x^{**} = average value in $\log_{10}$ $\pm s^{***}$ = standard deviation

Table 5 (A) Bacteriological and blood examinations of farm M. I (broilers)

Herd 76 without inulin, age of birds 1Week old

SAMPLE	TBC Cfu *	G negative Cfu *	Bdello. Pfu *	C. perf. Cfu *	P/BW g	BF/BW g	BW g	Endotoxin EU/ml	PC-BP (RE/ml)
1. Caecum	9.87	9.40	0.0	7	0.0019	0.0011	78.50	2.30	6.43
2. Caecum	10.04	9.95	0.0	7	0.0020	0.0010	72.00	2.50	3.80
3. Caecum	9.30	8.84	0.0	5	0.0028	0.0013	78.20	10.10	6.10
4. Caecum	10.18	9.87	0.0	6	0.0024	0.0014	62.30	2.50	5.53
5. Caecum	10.18	10.04	2.0	5	0.0024	0.0014	60.40	3.30	7.31
average value	9.91	9.62	0.4	6	0.0023	0.0012	70.28	4.14	5.83
s	0.37	0.18	0.90	1	0.0003	0.0002	8.58	3.35	1.31

Herd 75 with inulin, age of birds 1Week old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW g	BF/BW g	BW g	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	9.78	8.40	3.00	5	0.0032	0.0016	76.60	2.45	6.64
2. Caecum	10.0	9.18	3.00	5	0.003	0.0015	78.10	4.50	6.17
3. Caecum	9.18	8.84	4.00	5	0.0036	0.0018	77.30	4.60	4.73
4. Caecum	9.30	8.70	3.00	5	0.0032	0.0016	85.00	3.60	5.48
5. Caecum	10.18	9.98	3.00	5	0.0034	0.0017	87.70	5.30	2.55
average value	9.69	9.02	3.20	5	0.0033	0.0016	80.94	4.09	5.11
s	0.43	0.60	0.44	0	0.0002	0.0001	5.05	1.09	1.60

Herd 76 without inulin, age of birds 2Weeks old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW g	BF/BW g	BW g	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	10.65	10.00	0.0	6.0	0.0024	0.0014	150.10	3.00	8.05
2. Caecum	10.65	10.30	0.0	6.0	0.0027	0.0016	177.40	5.40	5.79
3. Caecum	10.54	9.84	0.0	7.0	0.003	0.0015	191.80	3.70	5.94
4. Caecum	10.90	10.40	0.0	5.0	0.0031	0.0015	116.90	3.70	6.14
5. Caecum	10.40	9.74	2.0	5.0	0.0028	0.0014	129.10	10.10	6.0
average value	10.63	10.07	0.4	5.8	0.0028	0.0014	153.06	5.18	6.38
s	0.18	0.29	0.90	0.84	0.0003	0.0001	31.55	3.02	0.94

Herd 75 with inulin, age of birds 2Weeks old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW g	BF/BW g	BW g	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	9.81	8.54	4.00	3.00	0.0051	0.0025	164.10	10.10	3.53
2. Caecum	8.90	8.70	3.00	6.00	0.0041	0.0022	111.90	2.40	2.66
3. Caecum	9.00	8´18	3.00	6.00	0.0051	0.00224	212.10	1.70	5.42
4. Caecum	9.18	8.18	4.00	5.00	0.0048	0.0025	192.60	1.00	2.89
5. Caecum	9.04	8.00	3.30	4.00	0.0049	0.0024	229.10	1.40	1.13
average value	9.19	8.36	3.46	4.80	0.0048	0.0024	181.96	3.34	3.13
s	0.36	0.32	0.51	1.30	0.0004	0.0001	46.01	3.82	1.55

Herd 76 without inulin, age of birds 3Weeks old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW g	BF/BW g	BW g	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	10.08	9.90	0	8.00	0.0045	0.0024	360	10.10	3.86
2. Caecum	9.93	9.90	0	8.00	0.0038	0.0021	250	1.80	5.15
3. Caecum	9.00	9.00	0	8.00	0.0029	0.0023	220	2.30	4.30
4. Caecum	8.90	8.81	0	6.00	0.0039	0.0024	320	2.20	2.61
5. Caecum	9.18	9.00	0	8.00	0.0037	0.0022	240	1.30	4.67
average value	9.42	9.32	0	7.60	0.0042	0.0023	278	3.54	4.12
s	0.55	0.53	0	0.98	0.0004	0.0001	59.3	3.68	0.97

* **Mean ± s of log $_{10}$ bacterial count/g**

Herd 75 with inulin, age of birds 3Weeks old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW g	BF/BW g	BW g	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	7.70	6.81	4.00	3.0	0.0046	0.0023	420	3.00	1.70
2. Caecum	7.87	6.93	4.30	3.0	0.0062	0.0029	290	2.20	2.80
3. Caecum	8.15	7.87	4.30	3.0	0.0052	0.0027	520	3.00	1.50
4. Caecum	7.84	6.04	3.78	0.0	0.0060	0.003	310	2.50	3.00
5. Caecum	7.70	6.48	5.00	0.0	0.0054	0.0028	320	3.10	6.20
average value	7.85	6.83	4.30	1.8	0.0055	0.0027	372	2.76	3.04
s	0.18	0.68	0.46	1.6	0.0006	0.0003	107.3	0.39	1.88

Herd 76 without inulin, age of birds 4Weeks old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW g	BF/BW g	BW g	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	9.54	9.00	0	7.00	0.0034	0.0019	800	1.40	6.12
2. Caecum	8.18	8.00	0	6.00	0.0041	0.0022	720	1.10	5.97
3. Caecum	9.30	9.30	0	7.00	0.0038	0.0019	430	1.60	4.50
4. Caecum	9.70	9.60	0	7.00	0.0036	0.0021	540	2.60	2.83
5. Caecum	8.70	8.30	0	7.00	0.0022	0.0013	470	10.10	0.96
average value	9.10	8.84	0	6.80	0.0037	0.0019	592	3.36	4.08
s	0.63	0.67	0	0.45	0.0003	0.0003	160.8	3.80	2.19

Herd 75 with inulin, age of birds 4Weeks old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW g	BF/BW g	BW g	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	9.30	7.40	4.54	5.00	0.0052	0.0026	520	1.30	4.15
2. Caecum	8.18	7.48	2.60	4.00	0.0102	0.0035	630	4.10	1.39
3. Caecum	8.70	6.90	4.81	4.00	0.0025	0.0019	680	1.90	2.46
4. Caecum	8.30	6.70	2.00	5.00	0.0035	0.0025	830	1.65	2.75
5. Caecum	7.70	7.18	4.60	4.00	0.0045	0.0025	780	4.30	3.80
average value	8.40	7.13	3.71	4.40	0.0052	0.0026	688	2.65	3.27
s	0.60	0.33	1.31	0.55	0.0030	0.0005	122.7	1.43	1.73

Herd 76 without inulin, age of birds 5Weeks old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW g	BF/BW g	BW g	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	9.30	8.70	2.00	5.00	0.0034	0.0017	1000	6.30	3.74
2. Caecum	9.18	8.48	0.00	5.00	0.0041	0.0019	840	2.10	5.13
3. Caecum	8.70	7.90	0.00	7.00	0.0038	0.0018	890	1.40	4.71
4. Caecum	8.70	8.40	2.00	5.00	0.0036	0.0016	800	2.75	3.56
5. Caecum	9.48	8.40	0.00	5.00	0.0022	0.0016	680	3.10	4.21
average value	9.07	8.40	0.80	5.40	0.0034	0.0017	842	3.13	4.27
s	0.36	0.29	1.09	0.89	0.0007	0.0001	117.5	1.88	0.66

Herd 75 with inulin, age of birds 5Weeks old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW g	BF/BW g	BW g	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	8.18	7.40	3.30	5.00	0.0036	0.002	1060	2.10	1.17
2. Caecum	7.18	6.30	3.00	5.00	0.0044	0.0024	1190	2.40	0.27
3. Caecum	7.30	6.30	4.18	3.00	0.0061	0.0029	1360	3.70	4.62
4. Caecum	8.90	6.60	4.00	3.00	0.0062	0.0028	1050	1.40	0.10
5. Caecum	8.90	6.30	4.30	3.00	0.0048	0.0024	1110	1.40	1.43
average value	8.09	6.56	3.76	3.08	0.0050	0.0025	1154	2.20	1.52
s	0.83	0.48	0.57	1.09	0.0011	0.0003	127.7	0.94	1.83

Herd 76 without inulin, age of birds 6Weeks old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW g	BF/BW g	BW g	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	8.48	8.18	0	6.00	0.0029	0.0029	1550	1.70	3.88
2. Caecum	9.18	8.48	0	6.00	0.0030	0.0030	1730	2.80	1.47
3. Caecum	8.60	7.48	0	7.00	0.0030	0.0030	1260	1.50	2.04
4. Caecum	9.30	8.65	0	6.00	0.0028	0.0028	1150	3.00	7.55
5. Caecum	9.00	8.40	0	8.00	0.0041	0.0041	1445	6.20	3.73
average value	8.91	8.17	0	6.6	0.0032	0.0032	1427	3.04	3.93
s	0.36	0.50	0	0.90	0.0005	0.0005	230.15	1.88	2.17

Herd 75 with inulin, age of birds 6Weeks old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW g	BF/BW g	BW g	Endotoxin EU/ml	PC-BP (RE/ml)
1. Caecum	7.18	6.60	3.60	4	0.0041	0.0041	1590	1.40	0.86
2. Caecum	7.18	6.60	4.00	4	0.0037	0.0037	1480	1.40	1.66
3. Caecum	7.30	6.00	4.00	4	0.0044	0.0044	1540	1.10	1.79
4. Caecum	7.70	6.30	3.00	4	0.0041	0.0041	1770	1.40	2.2
average value	7.34	6.38	3.65	4	0.0041	0.0041	1595	1.32	1.62
s	0.25	0.29	0.47	0	0.0003	0.0003	125.03	0.15	0.56

Table 6 (A) Bacteriological and blood examinations of I-group (H. 251) and C-group (H. 165)

Herd 165 without inulin, age of birds 1week old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW g	BF/BW g	BW g	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	8.40	7.30	0	0	0.003	0.0014	69.30	10.10	12.45
2. Caecum	8.70	8.00	0	0	0.0033	0.0014	65.80	6.80	19.78
3. Caecum	8.78	7.54	0	0	0.0037	0.0018	54.10	2.20	22.75
4. Caecum	8.95	8.70	0	0	0.0039	0.0019	61.90	1.60	10.92
5. Caecum	9.30	8.48	0	0	0.0035	0.0021	80.60	5.85	18.05
average value	8.83	8.00	0	0	0.0035	0.0017	66.34	5.31	16.79
s	0.33	0.60	0	0	0.0003	0.0003	9.77	3.50	4.98

Herd 251 with inulin, age of birds 1week old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW g	BF/BW g	BW g	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	9.30	7.30	0	0	0.0043	0.0023	80.60	4.2	11.45
2. Caecum	9.54	6.70	0	0	0.0042	0.0022	85.40	5.6	18.77
3. Caecum	9.40	7.91	0	0	0.0041	0.0021	65.00	5.5	12.55
4. Caecum	7.78	8.04	0	0	0.0041	0.0021	77.20	5.1	11.35
5. Caecum	9.30	8.41	0	0	0.0044	0.0021	74.70	4.7	17.44
average value	9.06	7.67	0	0	0.0042	0.0021	76.58	5.02	14.31
s	0.72	0.67	0	0	0.0001	0.0001	7.61	0.58	3.52

Herd 165 without inulin, age of birds 2Weeks old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW g	BF/BW g	BW g	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	9.00	8.60	1	4.00	0.0039	0.0025	228.20	3.60	18.4
2. Caecum	8.78	8.00	1	4.00	0.0036	0.0023	198.20	1.80	16.56
3. Caecum	9.08	7.30	1	3.00	0.0041	0.0021	235.30	2.30	14.4
4. Caecum	8.60	8.60	1	4.00	0.0031	0.0022	243.00	10.10	15.88
5. Caecum	9.00	8.30	1	3.00	0.0040	0.0019	196.20	10.10	20.72
average value	8.90	8.16	1	3.66	0.0037	0.0019	220.18	5.58	17.19
s	0.20	0.54	0	0.55	0.0004	0.0005	21.63	4.18	2.44

Herd 251 with inulin, age of birds 2Weeks old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW g	BF/BW g	BW g	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	9.15	7.00	3.18	0.00	0.0052	0.0026	245.50	5.80	10.35
2. Caecum	9.28	7.00	3.00	3.18	0.0055	0.0028	214.80	3.10	11.64
3. Caecum	9.32	7.00	3.00	0.00	0.0045	0.0020	263.00	9.90	8.55
4. Caecum	9.18	7.00	3.30	0.00	0.0047	0.0025	250.30	5.00	9.55
5. Caecum	8.23	6.00	4.00	3.00	0.0047	0.0025	230.00	1.10	9.00
average value	9.03	6.80	3.30	1.24	0.0051	0.0025	240.72	4.98	9.82
s	0.45	0.45	0.41	1.69	0.0005	0.0003	18.69	3.30	1.22

Herd 165 without inulin, age of birds 3Weeks old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW g	BF/BW g	BW g	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	8.04	8.38	1.60	5.00	0.0042	0.002	589	7.20	20.44
2. Caecum	7.70	8.70	0.00	5.00	0.004	0.0019	460	5.60	20.67
3. Caecum	8.08	8.30	1.60	6.00	0.0042	0.002	350	6.50	16.61
4. Caecum	8.70	7.70	0.00	5.00	0.0041	0.0024	500	5.10	22.64
5. Caecum	8.54	8.48	0.00	4.00	0.0037	0.0019	500	4.70	14.19
average value	8.21	8.31	0.64	5.00	0.0040	0.002	479.80	5.82	18.91
s	0.40	0.37	0.88	0.71	0.0002	0.0002	86.57	1.02	3.42

Herd 251 with inulin, age of birds 3Weeks old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW g	BF/BW g	BW g	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	7.70	6.04	4.00	4.00	0.0052	0.0027	590	10.10	5.78
2. Caecum	7.87	6.93	3.00	3.00	0.0055	0.0028	530	2.40	8.56
3. Caecum	8.15	6.87	4.00	4.00	0.0059	0.0028	640	1.70	6.97
4. Caecum	7.90	6.81	3.30	0.00	0.0055	0.0028	660	1.00	8.81
5. Caecum	7.70	6.48	3.48	4.00	0.0056	0.0025	610	1.40	8.13
average value	7.86	6.63	3.56	3.00	0.0055	0.00275	606	3.34	7.65
s	0.18	0.37	0.44	1.73	0.0002	0.0001	50.30	3.82	1.26

Herd 165 without inulin, age of birds 4Weeks old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW g	BF/BW g	BW g	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	9.18	8.40	0.00	6.00	0.0038	0.0019	880	3.00	18.66
2. Caecum	8.18	8.48	0.00	6.00	0.0039	0.0025	1110	10.1	35.64
3. Caecum	7.30	7.90	1.30	5.00	0.0036	0.0017	740	7.00	15.88
4. Caecum	8.70	8.70	3.00	6.00	0.0045	0.0023	730	3.00	20.67
5. Caecum	8.70	8.40	0.00	6.00	0.0045	0.0023	870	10.1	22.19
average value	8.41	8.38	0.68	5.80	0.0041	0.0021	866	6.64	22.61
s	0.71	0.29	1.32	0.45	0.0004	0.0004	153.39	3.55	7.66

Herd 251with inulin, age of birds 4Weeks old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW g	BF/BW g	BW g	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	8.70	6.4	4.00	3.00	0.0058	0.003	930	1.55	9.5
2. Caecum	8.40	6.48	3.00	3.00	0.0051	0.0026	1030	1.80	2.4
3. Caecum	8.70	6.90	6.00	3.00	0.0057	0.0031	1060	2.50	3.7
4. Caecum	9.30	6.70	5.30	4.00	0.0056	0.0024	940	2.40	4.0
5. Caecum	8.30	6.18	3.00	3.00	0.0058	0.0028	980	2.40	3.4
average value	8.68	6.53	4.26	3.20	0.0056	0.0028	988	2.13	4.6
s	0.39	0.28	1.35	0.45	0.0003	0.0003	56.30	0.43	2.8

Herd 165 without inulin, age of birds 5Weeks old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW g	BF/BW g	BW g	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	8.70	7.48	0	7.00	0.0039	0.0016	1340	10.10	21.36
2. Caecum	9.18	7.65	0	7.00	0.004	0.0025	1040	10.10	22.65
3. Caecum	8.70	8.87	0	7.00	0.004	0.0018	1470	10.10	25.5
4. Caecum	9.30	8.20	0	7.00	0.0038	0.0027	1170	10.10	25.51
5. Caecum	9.30	8.74	0	6.00	0.0039	0.0017	1190	10.10	21.94
average value	9.04	8.60	0	6.80	0.0039	0.002	1242	10.10	23.39
s	0.31	0.81	0	0.45	0.0005	0.0005	166.04	0.000	1.98

Herd 251 with inulin, age of birds 5Weeks old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW g	BF/BW g	BW g	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	8.18	6.60	4.00	4.00	0.0058	0.0029	1390	1.00	4.5
2. Caecum	8.18	6.00	3.30	3.00	0.0059	0.0029	1450	2.60	3.4
3. Caecum	7.30	6.30	3.00	4.00	0.0064	0.0033	1160	1.00	3.0
4. Caecum	8.70	6.60	3.00	3.00	0.0052	0.0028	1190	1.00	3.0
5. Caecum	8.70	6.30	3.40	3.00	0.006	0.0031	1300	2.20	3.4
average value	8.21	6.36	3.34	3.40	0.0059	0.003	1298	1.56	3.46
s	0.57	0.25	0.41	0.55	0.0004	0.0002	124.78	0.78	0.61

Table 7 (A) Bacteriological and blood examinations of farm R. I (broilers)

Age of birds 1week

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW	BF/BW	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	8.48	8.48	0.00	4.00	0.0029	0.0014	1.00	9.73
2. Caecum	8.65	8.00	2.00	4.00	0.0030	0.0015	1.60	8.14
3. Caecum	9.00	8.00	0.00	5.00	0.0030	0.0014	1.00	7.09
4. Caecum	8.95	7.70	2.00	5.00	0.0021	0.0013	1.00	7.37
5. Caecum	9.60	8.78	2.00	4.00	0.0027	0.0013	1.00	7.92
average value	8.94	7.99	1.20	4.40	0.0027	0.0014	1.12	8.05
s	0.43	0.30	1.09	0.54	0.0004	0.0002	0.27	4.01

Age of birds 2weeks

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW	BF/BW	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	8.41	8.20	0	5.00	0.0022	0.0012	4.70	2.37
2. Caecum	7.95	7.78	0	6.00	0.0020	0.0011	5.00	12.38
3. Caecum	8.14	6.65	0	6.00	0.0020	0.0010	4.40	23.71
4. Caecum	7.84	7.45	0	7.00	0.0020	0.0010	6.00	15.80
5. Caecum	8.74	8.20	0	7.00	0.0025	0.0011	3.70	30.69
average value	8.22	7.66	0	6.20	0.0021	0.0011	4.76	16.99
s	0.36	0.64	0	0.84	0.0002	0.0001	0.84	10.83

Age of birds 3weeks

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW	BF/BW	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	9.08	7.63	2.08	4.00	0.0033	0.0016	2.10	8.86
2. Caecum	7.97	7.95	2.64	6.00	0.0027	0.0013	2.30	9.72
3. Caecum	8.30	8.00	2.64	4.00	0.0035	0.0017	2.00	7.96
4. Caecum	9.04	8.80	1.70	4.00	0.0022	0.0013	3.45	8.66
5. Caecum	8.30	7.15	1.78	4.00	0.0033	0.0017	2.20	9.22
average value	8.54	7.90	2.17	4.40	0.0030	0.0015	2.41	8.88
s	0.50	0.60	0.45	0.89	0.0005	0.0002	0.59	0.65

Age of birds 4weeks

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW	BF/BW	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	7.80	6.90	4.00	0	0.0037	0.0019	1.00	14.73
2. Caecum	9.00	7.65	4.30	0	0.0041	0.002	1.00	0.10
3. Caecum	8.00	7.95	4.74	0	0.0040	0.0019	1.50	7.09
4. Caecum	8.44	8.40	5.30	0	0.0048	0.0022	2.50	5.37
5. Caecum	8.70	8.20	4.81	0	0.0043	0.0024	1.40	12.92
average value	8.39	7.82	4.63	0	0.0040	0.002	1.48	8.04
s	0.49	0.58	0.50	0	0.0004	0.0002	0.61	5.91

Age of birds 5weeks

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW	BF/BW	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	8.00	7.90	0	7	0.0037	0.0017	3.30	17.39
2. Caecum	8.65	6.98	0	7	0.0036	0.0018	3.30	0.10
3. Caecum	8.30	6.84	0	7	0.0047	0.0022	2.20	0.10
4. Caecum	8.48	8.30	0	7	0.0036	0.0019	3.35	11.14
5. Caecum	8.78	7.95	0	7	0.0037	0.0018	3.20	43.68
average value	8.40	7.60	0	7	0.0038	0.0019	3.07	14.48
s	0.30	0.64	0	0	0.0005	0.0002	0.49	17.93

Table 8 (A) Bacteriological and blood examinations of farm R. II (broilers)

Age of birds 5 Days old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW	BF/BW	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	9.48	9.48	2.00	3.00	0.0031	0.0015	2.00	5.17
2. Caecum	10. 65	9.00	1.00	3.00	0.0029	0.0016	2.10	4.45
3. Caecum	9.00	8.00	2.00	3.00	0.0022	0.0010	1.10	3.37
4. Caecum	9.95	9.18	1.00	5.00	0.002	0.0010	1.30	0.83
5. Caecum	9.60	9.00	2.00	3.00	0.003	0.0013	1.10	4.07
average value	9.50	8.93	1.60	3.40	0.0026	0.0013	1.52	3.58
s	0.39	0.56	0.54	0.89	0.0005	0.0003	0.49	1.67

Age of birds 10 Days old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW	BF/BW	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	9.41	8.20	2.00	4	0.0033	0.0015	2.00	0.52
2. Caecum	9.95	8.78	2.30	4	0.0037	0.0016	2.10	4.99
3. Caecum	8.54	8.00	2.00	4	0.0023	0.0013	1.10	2.00
4. Caecum	9.00	8.45	2.00	4	0.0032	0.0015	1.30	8.44
5. Caecum	8.74	8.20	2.30	4	0.0024	0.0014	2.10	3.49
average value	9.13	8.33	2.12	4	0.0030	0.0015	1.72	3.89
s	0.56	0.30	0.16	0	0.0006	0.0001	0.48	3.04

Age of birds 17 Days old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW	BF/BW	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	9.41	8.20	0	5.00	0.0028	0.0014	10.10	4.29
2. Caecum	8.95	8.78	0	5.00	0.0027	0.0013	3.40	4.74
3. Caecum	9.54	9.00	0	5.00	0.0027	0.0013	1.10	8.30
4. Caecum	9.84	8.45	0	6.00	0.0023	0.0011	1.60	8.62
5. Caecum	9.74	9.20	0	5.00	0.0031	0.0014	10.10	7.82
average value	9.50	8.73	0	5.20	0.0027	0.0013	5.26	6.75
s	0.35	0.40	0	0.47	0.0003	0.0001	0.54	2.07

Age of birds 22 Days old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW	BF/BW	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	7.62	7.00	3.70	5.00	0.0033	0.0015	4.80	6.30
2. Caecum	7.18	7.30	3.00	4.00	0.0047	0.0016	4.90	6.27
3. Caecum	7.60	7.30	3.30	3.00	0.0035	0.0013	8.10	10.54
4. Caecum	8.65	7.80	3.90	4.00	0.0030	0.0014	1.10	10.05
5. Caecum	7.30	7.00	3.00	4.00	0.0043	0.0014	1.10	0.12
average value	7.67	7.28	3.38	4.00	0.0029	0.0014	4.00	6.66
s	0.58	0.33	0.41	0.71	0.0003	0.0001	2.96	4.17

Age of birds 29 Days old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW	BF/BW	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	7.80	6.92	3.00	0	0.0041	0.002	3.50	3.04
2. Caecum	8.00	7.65	4.00	0	0.0043	0.0021	2.70	2.35
3. Caecum	7.00	6.94	3.00	0	0.0043	0.0021	3.30	2.36
4. Caecum	8.00	7.00	3.00	0	0.0042	0.0021	3.70	1.90
5. Caecum	7.30	7.20	4.00	0	0.0045	0.0022	1.10	2.97
average value	7.62	7.14	3.40	0	0.0043	0.0021	2.86	2.52
s	0.45	0.30	0.55	0	0.0001	0.0001	1.05	0.48

Table 9 (A) Bacteriological and blood examinations of farm Ro. (broilers)

Age of birds 2 weeks old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW	BF/BW	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	9.48	8.60	2.00	5.00	0.0041	0.0017	2.70	1.95
2. Caecum	9.54	8.74	2.00	5.00	0.0038	0.0018	3.30	4.70
3. Caecum	9.48	8.93	0.00	6.00	0.0042	0.0020	3.45	7.14
4. Caecum	9.9 0	9.32	2.00	5.00	0.0050	0.0021	10.10	3.70
5. Caecum	9.40	8.3 0	2.00	5.00	0.0048	0.0024	5.10	8.59
average value	9.47	8.90	1.60	5.20	0.0044	0.0021	4.93	5.22
s	0.45	0.31	0.89	0.45	0.0005	0.0002	3.02	2.66

Age of birds 3 weeks old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW	BF/BW	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	7.48	7.00	3.00	4	0.0043	0.0021	1.90	4.50
2. Caecum	8.54	7.74	3.00	4	0.0043	0.0026	10.10	2.12
3. Caecum	8.48	7.93	3.00	4	0.0046	0.0017	1.10	7.17
4. Caecum	8.90	7.30	3.00	4	0.0041	0.0014	1.10	0.90
5. Caecum	8.40	8.00	2.00	4	0.0041	0.0016	2.75	5.05
average value	8.36	7.60	2.80	4	0.0042	0.0019	3.39	3.95
s	0.53	0.43	0.45	0	0.0002	0.0005	4.42	2.48

Age of birds 4 weeks old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW	BF/BW	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	8.18	7.00	0.00	6.48	0.0032	0.0015	1.10	2.00
2. Caecum	8.54	7.74	3.00	5.00	0.0035	0.0029	1.10	2.32
3. Caecum	8.00	6.60	3.00	5.00	0.0041	0.0020	1.10	2.55
4. Caecum	8.48	7.48	2.00	5.84	0.0039	0.0022	1.10	2.80
5. Caecum	7.78	6.65	2.00	6.00	0.0029	0.0023	1.10	3.16
average value	8.20	7.10	2.00	5.66	0.0035	0.0013	1.10	2.57
s	0.32	0.50	1.22	0.65	0.0005	0.0005	0.00	0.44

Age of birds 5 weeks old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW	BF/BW	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	8.30	7.60	0.00	8.00	0.0025	0.0010	1.10	2.30
2. Caecum	8.00	8.00	0.00	7.30	0.0030	0.0010	1.10	2.44
3. Caecum	8.74	8.00	4.30	5.00	0.0031	0.0015	1.10	2.20
4. Caecum	8.48	7.60	4.00	5.00	0.0023	0.0010	1.10	2.80
5. Caecum	8.78	7.60	0.00	5..30	0.0023	0.0013	1.10	2.00
average value	8.46	7.66	1.66	6.32	0.0026	0.0011	1.10	2.38
s	0.32	0.22	2.27	1.56	0.0004	0.0002	0.00	0.33

Table 10 (A) Bacteriological and blood examinations of farm Co. (broilers)

Age of birds 3 weeks old

SAMPLE	TBC Cfu/g	G negative Cfu/g	Bdello. Pfu/g	C. perf. Cfu/g	P/BW	BF/BW	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	9.30	7.70	2.0	5.60	0.0036	0.0017	1.30	7.64
2. Caecum	9.54	8.74	2.0	5.30	0.0045	0.0018	2.90	6.47
3. Caecum	9.30	8.18	3.0	5.30	0.0037	0.0020	10.10	5.60
4. Caecum	9.90	8.78	3.0	5.60	0.0052	0.0021	3.20	3.19
5. Caecum	9.40	8.60	2.5	7.00	0.0041	0.0024	5.40	7.44
average value	9.49	8.40	2.5	5.76	0.0041	0.0020	3.58	6.07
s	0.25	0.46	0.5	0.70	0.0006	0.0003	1.02	1.80

Age of birds 4 weeks old

SAMPLE	TBC Cfu/g	G negative Cfu/g	Bdello. Pfu/g	C. perf. Cfu/g	P/BW	BF/BW	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	7.70	7.40	3.00	4.00	0.0033	0.0025	1.10	2.10
2. Caecum	8.78	8.00	3.00	3.00	0.0030	0.0014	1.10	2.10
3. Caecum	8.45	6.54	3.00	3.00	0.0032	0.0012	1.10	2.92
4. Caecum	8.65	7.11	3.00	4.00	0.0037	0.0014	1.10	2.2
5. Caecum	8.46	7.18	2.00	4.00	0.0040	0.0024	1.10	2.37
average value	8.41	7.25	2.80	3.60	0.0034	0.0018	1.10	2.34
s	0.41	0.54	0.45	0.54	0.0004	0.0006	0.00	0.34

Age of birds 5 weeks old

SAMPLE	TBC Cfu/g	G negative Cfu/g	Bdello. Pfu/g	C. perf. Cfu/g	P/BW	BF/BW	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	8.30	7.70	2.2	5.78	0.0034	0.0025	1.10	1.10
2. Caecum	8.54	8.18	2.2	5.00	0.0030	0.0010	1.35	2.17
3. Caecum	8.30	6.18	2.2	5.48	0.0030	0.0013	1.50	2.92
4. Caecum	7.90	7.78	2.2	5.30	0.0030	0.0013	1.10	1.59
5. Caecum	7.60	7.40	2.2	6.84	0.0024	0.0010	1.50	3.37
average value	8.13	7.45	2.2	5.68	0.0030	0.0010	1.31	2.23
s	0.37	0.76	0.0	0.71	0.0003	0.0006	0.20	0.93

Age of birds 6 weeks old

SAMPLE	TBC Cfu/g	G negative Cfu/g	Bdello. Pfu/g	C. perf. Cfu/g	P/BW	BF/BW	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	7.80	8.00	2.00	5.78	0.0021	0.0010	1.10	0.86
2. Caecum	8.20	7.40	1.30	6.60	0.0021	0.0010	1.10	2.17
3. Caecum	8.00	7.60	2.00	5.78	0.0022	0.0010	1.10	2.92
4. Caecum	8.55	7.81	2.00	5.48	0.0021	0.0010	1.10	1.59
5. Caecum	8.00	8.00	2.00	6.08	0.0029	0.0010	1.10	3.37
average value	8.10	7.76	1.86	5.94	0.0023	0.0010	1.10	2.18
s	0.28	0.26	0.31	0.42	0.0003	0.0000	0.00	1.01

Table 11 (A) Bacteriological and blood examinations of breeders (farm Ho.)

Age of birds 21 weeks old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW	BW gm	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	9.47	9.30	1.50	6.00	0.0012	1860	1	3.87
2. Caecum	8.69	7.69	3.00	5.00	0.0015	1710	1	2.75
3. Caecum	8.69	7.30	1.50	6.00	0.0016	2510	1	3.71
4. Caecum	9.54	9.00	2.00	5.00	0.0017	1870	1	3.62
5. Caecum	7.69	7.00	3.00	5.00	0.0011	1930	1	1.35
average value	8.82	8.06	2.20	5.40	0.0014	1976	1	3.06
s	0.67	0.92	0.75	0.54	0.0002	276.6	0	0.93

Age of birds 23 weeks old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW	BW gm	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	9.46	8.14	4.38	4.00	0.0021	2230	2.20	3.87
2. Caecum	9.17	8.07	4.65	4.20	0.0016	3120	2.00	2.75
3. Caecum	9.00	7.77	4.6	4.00	0.0021	2800	2.00	2.71
4. Caecum	9.04	7.60	3.44	4.00	0.0021	2130	1.60	3.62
5. Caecum	7.74	6.47	4.11	4.00	0.0015	2925	2.00	1.35
average value	8.88	7.61	4.23	4.04	0.0018	2641	1.96	2.86
s	0.59	0.60	0.49	0.08	0.0002	391.25	0.19	0.88

Age of birds 25 weeks old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW	BW gm	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	9.39	7.32	2	7	0.0019	2710	1.40	4.52
2. Caecum	8.07	7.23	2	7	0.0019	2450	1.30	5.89
3. Caecum	8.47	8.30	2	7	0.0015	2392	1.50	5.67
4. Caecum	9.39	6.69	2	7	0.002	2754	1.55	6.12
5. Caecum	8.00	7.87	2	7	0.0013	2250	3.40	5.10
average value	8.67	7.48	2	7	0.0017	2511.2	1.83	5.46
s	0.61	0.55	0	0	0.0002	192.17	0.78	0.65

Age of birds 27 weeks old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW	BW gm	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	6.90	6.74	3.07	4	0.0016	2925	7.60	5.30
2. Caecum	7.77	7.50	2.00	6	0.0011	2735	2.30	5.50
3. Caecum	9.07	9.00	2.30	6	0.0013	3550	2.00	5.67
4. Caecum	9.07	8.71	3.71	4	0.0011	2990	2.6ß	4.60
5. Caecum	9.39	8.85	3.34	5	0.0019	3110	1.9ß	5.30
average value	8.44	8.16	2.88	5	0.0014	3062	3.28	5.27
s	0.95	0.88	0.71	1	0.0003	272.5	2.17	0.41

Age of birds 29 weeks old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW	BW gm	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	8.60	6.65	3.30	4.0	0.0013	3050	2.60	4.11
2. Caecum	8.91	7.00	5.30	4.0	0.0015	3170	2.80	3.95
3. Caecum	8.60	8.54	5.30	4.0	0.0016	3700	3.00	3.16
4. Caecum	9.20	7.90	4.54	4.0	0.0014	2754	1.10	3.54
5. Caecum	8.54	7.30	3.60	6.0	0.0015	3970	3.40	2.44
average value	8.77	7.48	4.41	4.4	0.0014	3328.8	2.58	3.44
s	0.25	0.67	0.83	0.8	0.0001	443.23	0.88	0.67

Table 12 (A) Bacteriological and blood examination of breeders (farm Wa.)

Age of birds 34 weeks old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW	BW gm	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	9.46	8.97	1	8.0	0.0015	3210	9.7	35.17
2. Caecum	9.44	8.30	1	8.0	0.0011	3600	10.1	20.56
3. Caecum	8.38	7.87	1	7.0	0.0018	3440	9.4	20.15
4. Caecum	9.20	7.38	1	8.0	0.0012	3870	10.1	32.00
5. Caecum	9.43	7.49	1	7.0	0.0013	3710	10.1	22.34
average value	9.18	8.00	1	7.6	0.0013	3566	9.88	26.04
s	0.41	0.58	0	0.48	0.0002	226.68	0.32	7.023

Age of birds 36 weeks old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW	BW gm	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	9.69	6.72	3.36	7.00	0.0014	3290	4.56	13.38
2. Caecum	9.04	6.27	0.00	7.23	0.0011	4180	5.23	14.22
3. Caecum	10.07	7.04	0.00	7.00	0.0011	4300	5.67	12.03
4. Caecum	8.65	6.69	3.30	5.00	0.0015	4060	5.22	18.88
5. Caecum	9.17	6.14	3.54	5.00	0.0016	3430	5.44	19.78
average value	9.32	6.57	2.04	6.24	0.0013	3852	5.22	15.65
s	0.50	0.32	1.66	1.02	0.0002	411.21	0.41	3.45

Age of birds 38 weeks old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW	BW gm	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	9.60	7.65	1	7.00	0.0012	3776	2.00	16.16
2. Caecum	8.97	7.39	1	7.23	0.0017	3600	10.1	20.1
3. Caecum	8.95	6.00	1	7.00	0.0013	3590	2.70	21.15
4. Caecum	9.00	6.84	1	7.00	0.0015	3730	3.10	20.65
5. Caecum	8.60	6.77	1	7.00	0.0017	3620	10.1	36.31
average value	9.02	6.93	1	7.04	0.0014	3663.2	6.22	22.87
s	0.32	0.57	0	0.09	0.0002	75.37	3.88	6.94

Age of birds 40 weeks old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW	BW gm	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	7.17	6.69	3.3	5.00	0.0012	3690	1.10	5.34
2. Caecum	8.41	6.39	3.0	5.00	0.0012	3670	1.50	5.46
3. Caecum	8.47	8.30	1.5	6.00	0.0019	3570	1.90	4.64
4. Caecum	7.69	6.47	3.3	5.00	0.0016	3950	1.50	5.15
5. Caecum	7.54	7.00	1.5	6.00	0.0011	3160	1.90	7.11
average value	7.86	6.97	2.52	5.40	0.0014	3608	1.58	5.54
s	0.50	0.69	0.93	0.54	0.0003	256.8	0.29	0.93

Age of birds 42 weeks old

SAMPLE	TBC Cfu	G negative Cfu	Bdello. Pfu	C. perf. Cfu	P/BW	BW gm	Endotoxin EU/ml	PC-BP (RE/ml)
1.Caecum	9.47	7.39	2.30	6.00	0.0015	3660	2.10	10.34
2. Caecum	9.39	8.17	0.00	6.00	0.0016	4030	1.10	11.46
3. Caecum	9.87	7.84	5.30	5.00	0.0015	3840	2.10	11.64
4. Caecum	9.00	8.39	0.00	6.00	0.0013	4410	2.90	11.15
5. Caecum	9.00	7.17	4.30	5.00	0.0015	4063	2.30	10.88
average value	9.35	7.79	2.38	5.60	0.0014	4000.6	2.10	11.09
s	0.32	0.45	2.17	0.55	0.0005	250.66	0.64	0.51

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